EMBO reports

Distinct Regulation of Bioenergetics and Translation by Group I mGluR and NMDAR

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 April 2019

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee comments that is pasted below.

As you will see, all referees acknowledge that the findings are potentially interesting. However, they also all point out that the study will need to be carefully and significantly revised, and that the data need to be strengthened before it can be considered for publication here. Normally, I would ask for referee cross-comments at this point, given the long list of all comments. However, I will not be in the office next week and did not want to delay this decision further. If you like, we can discuss the revisions and requirements in more detail, and I can still ask for referee cross-comments, when I will be back in the office after the 29th of April.

At this point, given the overall very constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 6 main figures, I suggest that you layout the manuscript as a full article.

Supplementary figures, tables and movies can be provided as Expanded View (EV) files, and we can offer a maximum of 5 EV figures per manuscript. EV figures are embedded in the main manuscript text and expand when clicked in the html version. Additional supplementary figures will need to be included in an Appendix file. Tables can either be provided as regular tables, as EV tables or as Datasets. Please see our guide to authors for more information.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution. In order to avoid delays later in the process, please read our figure guidelines before preparing your manuscript figures at: http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In their manuscript 'Distinct Regulation of Bioenergetics and Translation by Group I mGluRs and NMDARs', the authors set out to investigate which biological processes are responsible for the vast ATP consumption of the brain. They find that protein synthesis in response to glutamate stimulation is a major consumer of ATP. As glutamate activates several receptors, the author investigated whether mGluR and NMADR had different effects on ATP consumption and protein synthesis, and they found that mGluR stimulation caused rapid protein synthesis while NMDAR stimulation did not affect ATP levels or protein synthesis in the same time window. The authors identified AMPK

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as the switch downstream of the glutamate receptors: mGluR stimulation caused inhibition of AMPK and activation of eEF2, while NMDAR stimulation lead to phosphorylation of AMPK and eEF2. Overall this is an interesting story that provides mechanistic insight into the regulation of protein synthesis downstream of different glutamate receptors and makes a compelling case for activity-induced protein synthesis as a major contributor to the high ATP consumption in the brain. The experiments use appropriate methods, are well documented and for the most part carefully analyzed.
There are some points that ought to be addressed before publication.
Major points:
Fig. 1E: The synaptosomal prep is not convincing. GFAP should be absent in a proper SN prep.
The authors are careful to provide all statistic details and use appropriate test throughout. However, the Ns vary greatly between figures and sometimes even within figures. For example in Figure 1F, the number of data points per bar ranges from 5 -10. The last two bars (NMDA, NMDA+AP5), have the lowest number as a consequence a much high variance than the other bars. Not surprisingly then, the authors did not find any statistical differences for the NMDA conditions. They authors should use the same N for all conditions and/or perform a power analysis to justify their chosen n values.
In Fig 2C-E, the authors focus their statistical analysis solely on differences between the bars. However, the primary question of these experiments was whether any individual treatment had an effect. The author should compare the means of each bar to the hypothetical value 0 (One sample t test or Wilcoxon signed rank test).
Fig. 4E: the author propose that de-novo synthesis of alpha-SNAP is required for AMPK dephosphorylation. Can they directly prove this point, for example by using siRNA against alpha-SNAP?
Minor points:
The manuscript would profit from careful copy-editing.

Referee #2:

This manuscript provides evidence that protein synthesis triggered glutamate treatment largely occurs through mGluR activity, and that this is a (and maybe the) major consumer of ATP in cultured neurons. This is an important area of research, and the proposed finding is very interesting. The experiments appear to have been done very carefully. My major concern is with the some of the data analysis and the some of the conclusions reached by the authors as well as the functional state of the neuronal cultures.

Major concern 1
Well regarded have shown that ATP degradation is largely due to ion transporter activity following action potentials, and vesicle recycling. Therefore, the finding presented here, that protein synthesis is the major consumer of ATP, now provides a third mechanism to consider. However, it's concerning to me that in the work presented here, the only consumer of ATP is protein synthesis. This suggests that something is fundamentally different in the experimental preparations or the way the experiments were performed. Concerning the preparation, one possible explanation is that the cultured neurons here have no or low basal synaptic and action potential (AP) activity. If so, then application of agonist is effectively stimulating an otherwise inactive low activity neuronal culture, which could trigger protein synthesis. Part of my concern is if the cultured neurons had been active before addition of agonist, the relative change in protein synthesis levels may not be significant.

My minimum recommendation to address the concerns outlined above is that the action potential kinetics (shape and amplitude), threshold, and firing frequency, all need to be measured for the cultured neurons and the values need to be in the range of physiological levels. In addition, it needs to be determined that synaptic transmission occurs in the cultured neurons, and that transmitter release can follow physiological firing frequencies. In cultures where all of the AP and neurotransmitter release parameters match physiological levels, then the experiments shown in figure 1 A,B,C and figure 2 need to be repeated to establish that the data shown in these figures can be repeated in a fully functional cell line. For figure 2, it is also necessary to show ATP:ADP changes in the soma and axon hillock to determine if the results shown in figure 2 are specific to the dendritic regions. This will also verify that the batch of PercevalHR being used, and the experiments are sensitive and rapid enough to detect changes in ATP levels that are necessary to restore ion concentration gradients. This would then allow a direct comparison of ATP usage in different

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neuronal compartments to verify the explanation that is given in lines 555 to 558. This direct comparison of different compartments is also relevant to the statements in lines 56 to 60. Lastly, the ATP:ADP ratios shown in this manuscript appear to be much smaller than those shown in ref 8 from your manuscript. Please discuss this.

Major concern 2
Another possibility to explain the results is that protein synthesis is necessary to initiate or maintain another process that consumes energy, such as transporter activity, vesicle release + recycling, etc. One possible method that could address this concern is to use a non-canonical amino acid that produces non-functional proteins. This would allow protein synthesis to occur and therefore consume energy but it would block any separate activity that consumes energy by the newly synthesized proteins.

Major concern 3
In many experiments shown here, n-values were too low. In all bar graphs, the actual data points should be shown (as they are for the bar graphs in figures 1 and 2). In some cases, a single data point or two appear to be largely responsible for affecting statistical significance levels. For example, the single data point at 1.2 for Glut+MPEP for Fig 1C. Finally, when you make a statement that treatment values are not different from basal conditions, you should directly compare them. For example, Glut+MPEP in Fig 1C should be also be compared to basal levels to determine significance levels.

Additional concerns, by section or line by line:

Suppl. Figures: please remove numbering in the margins
Line 99: glycine should be added as a coagonist to activate NMDA receptors. This has potential relevance to the rest of the paper
Line 105-106: this needs to be reworded (inhibitors applied in the absence of glutamate....)
Line 117: statistics need to be given
Line 126: NMDA impact (see concern given for line 99, co-agonist)
Line 126: provide stats for NMDA vs Basal, and also NMDA vs DHPG
Line 131: provide stats for Dynasore vs Glut, and also Glut vs Glut+Dynasore
Line 137: These synaptosomes have the same level of GFAP as whole cortex lysate, therefore there is a lot of glial material in your synaptosomes. Other papers show a reduction in GFAP in synaptososme preps. For ex. see J Neurosci Methods. 2006 Nov 15; 158(1): 30-36.
Line 182: Did you measure change in the entire region shown in fig 2A? If you used a region of interest, please show the ROI.
Line 191: Fig 2B, is the y-axis ATP:ADP?
Line 213-214: please reword for better explanation
Line 206-217: Figs 2G and 2H stats appear to be comparisons with respect to response at stim onset. Please also compare DHPG vs DHPG+Aniso at their matching time points.
Line 270-274: If there is labeling before stimulation, this means that there is a basal level of protein synthesis. The finding that NMDA treatement decreases the Funcat signal means that existing protein synthesis is somehow decreased, and proteins labelled during the incubation period have been destroyed. This doesn't make sense to me. Please provide an explanation for these results to explain this.
Line 273: Please show each data point for figure 3D. Based on the error bars, the statistical significance levels appear to be unusually high. The same for figure 3F.
Line 314-315: Since the 1 min stimulation data is not shown, this should be removed from the protocol in fig 3A.
Line 566-567: synaptic is presynaptic and postsynaptic compartments, but the presynaptic energy use has not been adequately addressed here. Only dendritic, so saying synaptic can be confusing here.

Referee #3:

Review of Datidar
The authors have some very interesting findings that inhibiting translation can block the downlregulation of ATP levels seen after depolarization that corresponds to an increase in

translation. They then conduct some correlational studies of phosphorylation in other pathways (AMPK, eEF), and some pharmacology to try and define the pathway. Further, they show that this induction of translation is mediated more immediately by mGluR's than by NMDA receptors. So there are interesting components. But, there are some major concerns that seem difficult to overcome in the papers current form.

Major scientific concerns:
• Claims include "little is known about the processes within neurons responsible for such abundant ATP outlay" which is counter to the well know processes of neuronal polarization, for example, that is well documented to be highly energetically demanding. Thus, it is very interesting that in response to a Glu stimulation, they authors can block changes in ATP by inhibiting translation. To put these results in context, however, they need to do a better job of reviewing the literature (including their ref 7). For example, they contrast their Chx findings with a block of vesicular recycling (which, according to their ref 7 is a GTP consumer rather than ATP), but don't include a control/comparison to inhibiting the processes that are thought to be the most consumptive (e.g. the Na/K ATPase that maintains ion gradients). They don't confirm that Chx or Aniso don't hit these other targets. Thus, they need to include more thorough scholarship in the introduction and perhaps some additional controls to put their major findings in context
• ATP levels are shown to be altered and thus the authors assume this is because of changes in ATP usage. However, ATP levels are a function of both ATP consumption an ATP production. Thus, any decrease in ATP could equally be due a slow down in ATP production rather than an increase in consumption. Indeed, ref 7 reviews literature about neuronal activity rapidly inducing mitochondrial ATP production. The authors should conduct experiments to determine whether their findings reflect changes in production, consumption, or both.
• The timing of the phenomena they study don't cleanly fit to their model that translation is driving loss of ATP? How do the authors reconcile the rapid inhibition of protein translation through activation of NMDA receptors which is referenced (lines 68-69, ref 16) as well as confirmed in Figure 3B seen at 2min post-stimulation with the unchanged ATP measurements following NMDA stimulation? If NMDA treatment induces an inhibition of protein translation (looks about 35% in fig 3B) and the claim is that protein translation is a major consumer of ATP within the postsynaptic density, why is there not an increase in available ATP in the NMDA-treated relative to basal conditions? If this is simply because the time points in fig 1 are too late, the experiment should be replicated at the 2 minute time point.
• Key experimental details are missing from this manuscript which makes it extremely difficult to assess the veracity of statements and conclusions that the authors are making. Some of these omissions include the Mg2+ concentrations in experiments assaying NMDAR function, the timing of when protein synthesis inhibitors are added to the media, and why several of the immunoblots displayed show tubulin twice and levels of tubulin are changing between the two blots (within the same condition, same time point) .
• If all of the above work out, AMPK canonically responds to AMP levels. Therefore, the authors should also be considering the possibility that the AMPK activation is actually downstream of the ATP consumption. Are their data equally consistent with a model where ATP is consumed after Glu stimulation, and this is what then leads to elevated AMPK activity - in other words, in their summary figure should AMPK be a different position?
Other concerns by line and figure
• Line 376: the claim " indicating mGluR dependent de-novo synthesis of a-SNAP plays a critical role in AMPK dephosphorylation" is overstated. To make this claim, the need to specifically remove a-SNAP or its signaling capacity, rather than inhibiting synthesis of all proteins.

• Is AMPK supposed to phosphorylate eEF2 directly? This is a bit unclear in the writing in the results. It is made a bit more clear in discussion that they think this is largely indirect. Line 479: the use of the word 'direct' here implies AMPK is phosphorylating eEF2. But I don't the authors have support for that statement. They would need more biochemical evidence to establish a direct relationship than is provided here. They should perhaps rephrase more carefully.

• Line 346: The authors should introduce the direction of effect of ATP/AMP ratio on AMPK phosphorylation/activation.

Figure 1
• It would be helpful for the authors to add an experimental timeline, per Fig. 2F or Fig. 3A.

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Specifically, authors should state when the protein synthesis inhibitors were added (i.e. pretreatment or co-applied with glutamate/other agonists); and, for the cortical neuron experiments (Fig. 1A-C), clarify whether the ATP levels were measured immediately after the glutamate treatment, or after a recovery period.
o We would like to also see how each of the treatment conditions, and particularly protein synthesis inhibitors if they are added ahead, might change cell surface receptor expression of NMDAR, AMPAR, and mGluR within a treatment time window that matches the experimental time frame used in this and other figures.
• In the figure legend, the text, or in the Methods section, there isn't a clear explanation of why and how and why the "relative ATP level" was used for the synaptosome experiments in Fig. 1D & F, especially when a different normalization is used for cell culture experiments (ratio of ATP / Total ATP + ADP).
• They conclude from Figure 1 and Supplementary figure 1 that vesicle endocytosis has negligible effect on pan-neuronal ATP level (lines 130-132; mentioned again in the conclusion, lines 555-557), countering the work of Rangaraju et al 2014 (ref 16). However, this is misleading in that their experimental design does not examine presynaptic vesicle endocytosis from synaptic vesicle cycling during synaptic transmission, as application of glutamate & other receptor agonists only involves postsynaptic terminals. Therefore, while it may be that protein synthesis is a major ATP consumer in the postsynaptic neuron, it is unknown how this compares to ATP consumption in the presynaptic neuron based on the current design.
• Fig 1F -- see comment on Mg2+ block and NMDAR activation.

Figure 2
• Throughout the study, the authors use NMDA to activate NMDA receptors in cultures and synaptosomes, but do not mention the concentration of Mg2+ present in media/buffer. If such solutions were used, it should be mentioned here and in the methods. If not, the effect of removing the Mg2+block on kinetics of ATP consumption and NMDAR-activation-mediated protein synthesis should be examined. (i.e. repeat experiments in Mg2+ free buffer/medium).
• The authors should mention when the protein synthesis inhibitor was added (pre-incubation before addition of NMDA/DHPG or simultaneous coadministration), and
• Does Anisomycin alone show any effect on the ph-corrected Perceval Ratio? The authors should show these data. One can imagine that a translation inhibitor could effect an assay dependent on an overexpressed protein construct.
• The change in ATP/ADP ratio in unstimulated and anisomycin-only-treated conditions should be moved to the main figure (2G and 2H) from Supplementary Figure 2, and included in the statistical analysis of Fig 2C-E.

• Correct figure 2E legend to "10 minutes" from "2 minutes."
• Figure 2B seems to call for a repeated measures ANOVA

Figure 3
• In Figure 2, it was suggested that activation of NMDAR induces delayed protein synthesis (5-10 minutes). The results from Fig 3A-D suggest that NMDAR activation induces a rapid inhibition of translation. It would be interesting to see whether there is de novo protein synthesis after 10 minutes of NMDA stimulation.
• Data in Fig 3D could be annotated better (No explanation of B2, D2, N2, etc in figure legend or text) or be removed altogether, as it is the same information as 3C.
• In Fig 3B and E, add a zoomed-in inset of images at the dendrite to visualize protein synthesis/eEF2 phosphorylation at the dendritic level.
• 3B-3C and 3E-3F, graphed data is normalized to Map2 intensity. Please show these Map2 images in the main figure adjacent to the peEF2 images.
• Fig 3H: further clarify how the relative eEF2 phosphorylation levels were quantified here ((p-eEF2/ Tubulin) / (initial p-eEF2 / initial tubulin)?.
• Fig 3H should also include the trace for the unstimulated condition (Supplementary figure 4B), include discussion of the increase in phosphorylation seen in the unstimulated condition over time. The statistical analysis done here should include the unstimulated condition (also increase sample size for unstimulated? "n{greater than or equal to} 2 animals per group")
• Fig 3H It's not clear why two Tubulin blots are shown for each stimulation set. Furthermore, at some time points (DHPG @ 2 mins, 5 mins), Tubulin levels are changing relative to each other more so than p-eEF2/T-eEF2 levels.

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Figure 4
• A better representative sample image for DHPG + Aniso in Fig 4C is needed where the lanes are not overlapping.
• Fig 4D & F should include traces from Unstimulated & Unstimulated + Aniso to show the effects of protein synthesis inhibition on AMPK phosphorylation & alpha-SNAP synthesis.
• Clarify which condition the images in Fig 4G is from, and include representative images from both basal and DHPG conditions + some zoomed in images.
• 4A-4B, graphed data is normalized to Map2 intensity. Please show these Map2 images in the main figure adjacent to the peEF2 images because the values shown in the graph suggest that the chosen images have vastly different Map2 expression levels, which probably indicates they are not faithful representative images.
• Figure 4H - correlation needs to be calculated w/in the cell. Not the whole image. Otherwise the finding might just reflect the difference between the inside and outside of the cell. More importantly, this kind of imaging is supportive that the proteins could be interacting, but does not have the resolution to say they are associated, as suggested on line 378. True molecular interactions would need to be shown by Co-IP or Fret, or other appropriate assay.

Figure 5
• 5A: again, not clear why two tubulin blots are shown and why they are discordant.
• 5C-E: Need some sort of color legend/scale.
• 5C-5G: Is this novel? That NMDA receptor activation leads to rapid calcium influx has been known for quite some time; not only that, the calcium imaging experiment doesn't directly tie NMDAR-mediated rapid calcium influx to AMPK activation. Should be supplemental info if authors are just showing that they can induce calcium influx in neurons in response to nNMDA in their hands.
• Methods section states that Fmax was calculated with 10uM ionomycin and 2.5 mM Ca++. The figure states 10 mM Ca; authors should clarify which condition was used for Fmax calculation.
• Figure legend for 5G should state the time point for when ΔF/F0 was measured (i.e. 5 minutes into treatment, average of 30 sec).

Figure 6
• Map2B stain in Supplementary figures should be shown with the representative images in 6A, C, E, G.
• So here, the authors state that the AICAR and CC were applied for a full hour before receptor agonists were added (5min). It is difficult to tell whether this was also the case for the other treatments from previous experiments. Again, adding a timeline of treatment here would be helpful.

Finally, there are a very large number of typos (1-2 per para) that would preclude publication in its current form. Here are a subset:
"while mGluR leads" in abstract.
"ischemia lead" in intro
"on synaptic activity" seems to be missing a word. (.e.g upon induction of synaptic activity, or by synaptic activity) in intro
"mutually exclusive mechanisms" in intro
Spelling of significant in the first para of results.
Do the authors indeed mean "Bonferroni's multiple comparison test" in figure legends. Mostly Bonferroni's is used to mean a correction applied after tests, rather than a test per se.
543: lines of evidence
Verb conjugation has frequent mistakes throughout.
No references for the statement that fMRI and PET studies have established correlations between activity and ATP consumption.
References 4 &5 do not support the contention that metabolism is at the heart of these conditions.
Ref 16 is incomplete
815: for non parametric data, I assume?
587: correlates instead of collates?
Line 556 has more commas than needed.
Why are weblinks found in references sometimes? This does not seem standard.

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Response to Reviewer's Comments

#Reviewer 1:

We thank the reviewer for finding out story 'interesting that provides mechanistic insight' in the context of protein synthesis regulation by various glutamate receptors. We have now tried to improve our manuscript by carefully addressing the remaining comments.

Major Concern 1: Related to Figure 1-E
"The synaptosomal prep is not convincing. GFAP should be absent in a proper SN prep."
Response: In the current work, we have deliberately chosen the relatively crude method of synaptosome preparation (by filtration) since the synaptoneurosome prepared by more elaborate method has lower success rate in responding to neurotransmitter stimulation. This is a well-established method to rapidly isolate functional synaptoneurosomal particles as described by Hollingsworth et. al, 1985. This method has been adopted by various other groups to understand synaptic components and the effect of synaptic activity (1)(2)(3). The method is relatively crude in nature as compared to other percol-density gradient centrifugation-based methods. But these elaborate preparations suffer from the problem of producing significant fraction of non-functional particles which are problematic to properly quantify the effect of stimulation.

Because of its crude nature, the preparation does not show significant de-enrichment of glial marker GFAP. But this contaminating protein could be very likely contributed by cell debris or broken fragments passed through the filter pores as majority of the intact particles in the preparation are seem to be 'snow-man' shaped synaptoneurosomes as represented by the electron micrograph images (Supplementary figure 1F). Significant enrichment of pre-synaptic marker protein Synapsin 1 and post-synaptic marker PSD 95 also further supports this (Figure 1G). Therefore, we believe that the activity induced changes in our various synaptoneurosomal read-outs are majorly due to synaptic compartments.

"The authors are careful to provide all statistic details and use appropriate test throughout. However, the Ns vary greatly between figures and sometimes even within figures. For example in Figure 1F, the number of data points per bar ranges from 5 -10. The last two bars (NMDA, NMDA+AP5), have the lowest number as a consequence a much high variance than the other bars. Not surprisingly then, the authors did not find any statistical differences for the NMDA conditions. They authors should use the same N for all conditions and/or perform a power analysis to justify their chosen n values."

Response: We thank reviewer for the suggestion. We have now increased the number of experimental replicates and made sure every group has 8 data points as opposed to previously reported minimum 5 data points. This helped in keeping uniformity across groups as suggested by the reviewer. Also, to clearly understand the effect of mGluR and NMDAR, we have separated the analysis receptor wise i.e. NMDA and NMDA+AP5 treatment effect is now compared to its unstimulated basal separately from mGluR related groups (Figure 1H and 1I). Even after all these analyses, the pattern and the statistical significance remains the same between two groups being compared as reported in the previous version of the manuscript. To help understand the statistical comparison, we have provided the statistical details in the table if statistical analysis separately.

We have avoided the use of power analysis because post-hoc power analysis computes the sample size for a certain experimental design for a specific level of significance and effect size. The effect size is a scientific question that we are trying to address with our experiments. Power analysis also requires information about various parameters that can affect the test statistics. Therefore, without the knowledge of all these factors, computing sample size through power analysis could be misleading.

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Major Concern 2: Related to Figure 2C-E
"In Fig 2C-E, the authors focus their statistical analysis solely on differences between the bars. However, the primary question of these experiments was whether any individual treatment had an effect. The author should compare the means of each bar to the hypothetical value 0 (One sample t test or Wilcoxon signed rank test)."

Response: We recognize the importance of the suggestion by the reviewer to investigate the statistical significance of individual treatments. We have now compared the individual groups with the basal group. The test statistics has been included in the manuscript in table of statistical analysis (Supplementary Figure 2C-2E).

Major Concern 3: Related to Figure 4E
"The author propose that de-novo synthesis of alpha-SNAP is required for AMPK dephosphorylation. Can they directly prove this point, for example by using siRNA against alpha-SNAP?"

Response: We have now completed the experiment suggested by the reviewer. We knocked down alpha-SNAP with specific siRNA and compared the effect of DHPG stimulation on AMPK phosphorylation with the cells treated with scramble siRNA. Our observations indicate that knocking down alpha-SNAP completely abolishes the DHPG induced dephosphorylation of AMPK in cortical neurons (DIV 15) therefore; substantiating the crucial role of alpha-SNAP on DHPG induced AMPK regulation (Figure 4I).

#Reviewer 2:

We thank the reviewer for acknowledging the importance of our work and finding our manuscript 'interesting'. In the following section we tried to address all the remaining comments and concerns.

Major Concern 1:

"Well regarded have shown that ATP degradation is largely due to ion transporter activity following action potentials, and vesicle recycling. Therefore, the finding presented here, that protein synthesis is the major consumer of ATP, now provides a third mechanism to consider. However, it's concerning to me that in the work presented here, the only consumer of ATP is protein synthesis. This suggests that something is fundamentally different in the experimental preparations or the way the experiments were performed. Concerning the preparation, one possible explanation is that the cultured neurons here have no or low basal synaptic and action potential (AP) activity. If so, then application of agonist is effectively stimulating an otherwise inactive low activity neuronal culture, which could trigger protein synthesis. Part of my concern is if the cultured neurons had been active before addition of agonist, the relative change in protein synthesis levels may not be significant.

My minimum recommendation to address the concerns outlined above is that the action potential kinetics (shape and amplitude), threshold, and firing frequency, all need to be measured for the cultured neurons and the values need to be in the range of physiological levels. In addition, it needs to be determined that synaptic transmission occurs in the cultured neurons, and that transmitter release can follow physiological firing frequencies. In cultures where all of the AP and neurotransmitter release parameters match physiological levels, then the experiments shown in figure 1 A,B,C and figure 2 need to be repeated to establish that the data shown in these figures can be repeated in a fully functional cell line."

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Response: We appreciate this insightful comment from the review. We want to clarify that our work does not contradict the proposal that vesicle recycling and action potential would consume considerable amount of energy while points out that protein synthesis downstream of synaptic activity also has a significant contribution to this (Supplementary Figure 1B and 1C). We are now providing evidence to show the cultured neurons we have used are healthy, electrically active and are comparable to the previous reports (Figure 1D, 1E, Supplementary Figure 1H, 1I, 1J and 1K).

"For figure 2, it is also necessary to show ATP:ADP changes in the soma and axon hillock to determine if the results shown in figure 2 are specific to the dendritic regions. This will also verify that the batch of PercevalHR being used, and the experiments are sensitive and rapid enough to detect changes in ATP levels that are necessary to restore ion concentration gradients. This would then allow a direct comparison of ATP usage in different neuronal compartments to verify the explanation that is given in lines 555 to 558. This direct comparison of different compartments is also relevant to the statements in lines 56 to 60. Lastly, the ATP:ADP ratios shown in this manuscript appear to be much smaller than those shown in ref 8 from your manuscript. Please discuss this."

Response: We thank the reviewer for this suggestion and agree this has improved our analysis significantly. As per suggestion we quantified the ATP:ADP ratio at the somatic compartment on both stimulations (Figure 2D). When quantified only in somatic compartment, we observed no significant change in the ATP:ADP ratio on either of the stimulations compared to unstimulated group. These observations indicate that the stimulation dependent changes in ATP:ADP ratio as shown in Figure 2E is dendrite specific. Therefore, our observations from Figure 2D and 2E together strongly suggest a robust compartmental regulation of bioenergetics on stimulation within neuron.

While the observations for compartmental energy regulation was interesting, unfortunately we were unable to quantify ATP:ADP ratio at the axon hillock as it would require over-expression of an axon hillock specific marker protein to identify the region. This would need an additional laser line and relevant fluorescent protein to image. Since, the PercevalHR and pHRed uses 4 laser channels already, it was not feasible for us to do the experiment.

To test the sensitivity of PercevalHR, the same batch was further used in Glutamate stimulation experiments (Supplementary figure 2I) as well. In those experiments we tested the sensitivity of the reporter by treating the cells with metabolic inhibitor deoxy-glucose which caused significant reduction in the cellular ATP:ADP ratio and therefore Perceval fluorescence within minutes. Subsequently, rapid change in ATP:ADP ratio detected by the change in fluorescence on glutamate stimulation corroborates that it is fast enough to detect the rapid changes in ATP levels. The sensor time constant for ATP unloading and ADP unloading is reported to be ~2.1s and ~1.5s at 25°C by (4). Therefore, the sensor is expected to report changes in the few seconds time scale within the mammalian neurons.

Lastly, in ref 8 Rangaraju et. al, 2014 did not quantify ATP:ADP ratio.

1) Their experimental reporter was designed to report ATP levels directly using luminescence-based principles. The sensitivity of such sensors could be different than fluorescence ratiometry based sensors like Perceval.
2) Their experiment involved electrically stimulating cells for AP firing for 60s with 10Hz. This is in contrast to our protocol where we applied the chemical agonists in bath. So, we feel comparing these two cases directly in terms of the relevant read-outs can be misleading.
3) Their measurement is solely Pre-synaptic by nature.
4) Authors in ref 8 have repeatedly mentioned about the little effect of AP stimulation on the energetics of intact nerve-terminal. They went to explain how activity dependent ATP synthesis gives robustness to the steady-state energy level which we have also observed in our metabolic inhibition experiments. In fact, ref 8, 9, 17 all of them show modest effect of stimulation on steady state energy level which is probably important for the physiological function to continue without putting the cell under energy stress.

Major concern 2

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"Another possibility to explain the results is that protein synthesis is necessary to initiate or maintain another process that consumes energy, such as transporter activity, vesicle release + recycling, etc. One possible method that could address this concern is to use a non-canonical amino acid that produces non-functional proteins. This would allow protein synthesis to occur and therefore consume energy but it would block any separate activity that consumes energy by the newly synthesized proteins."

Response: The reviewer presents an interesting possibility. However non-canonical amino acids have been shown to incorporate into proteins without changing their folding or targeting to their specific areas (5). Furthermore in a recent publication Schuman and colleagues adapted the NCAT labeling in vivo in mice and synthesis of non-functional proteins would have compromised animal health (6). Even the accumulation of non-functional proteins would also bias the energy utilization to processes to break down said proteins or set up an unfolded protein response compromising cell health. We will incorporate the possibility raised by the reviewer in the discussion which will no doubt enhance the interpretation of the paper.

Major concern 3

"In many experiments shown here, n-values were too low. In all bar graphs, the actual data points should be shown (as they are for the bar graphs in figures 1 and 2). In some cases, a single data point or two appear to be largely responsible for affecting statistical significance levels. For example, the single data point at 1.2 for Glut+MPEP for Fig 1C. Finally, when you make a statement that treatment values are not different from basal conditions, you should directly compare them. For example, Glut+MPEP in Fig 1C should be also be compared to basal levels to determine significance levels."

Response: We have increased the n numbers to the wherever it is possible within the time limit given for the revision. We now have provided an extended data table for all the analysis and the statistics done on all the data tables in all figures to reach relevant conclusions. We have also added the comparison analysis of basal group with various other treatment groups as suggested by the reviewer and have tried avoid use of confusing sentences in our conclusions (here we also request the reviewer to refer to our response to the reviwer#1 comment on the similar question).

Additional Concerns:

Suppl. Figures: please remove numbering in the margins
Response: We apologize for the inconvenience. We have removed the margins.

Line 99: glycine should be added as a co-agonist to activate NMDA receptors. This has potential relevance to the rest of the paper
Response: In our experiment we have followed the protocol which is known to induce NMDA dependent LTD in hippocampus (7)(8)(9)(10). Doing these experiments with Glycine to elicit NMDAR response (which could also be further tested with other NMDAR modulators such as dopamine) would be very interesting and could be quite different from NMDA as an agonist alone. But we feel that at the moment those would be beyond scope of the current manuscript.

Line 105-106: this needs to be reworded (inhibitors applied in the absence of glutamate....)
Response: We have altered the sentence as suggested by the reviewer.

Line 117: statistics need to be given
Response: We have added the detailed statistics on table of Statistical Analysis.

Line 126: NMDA impact (see concern given for line 99, co-agonist)

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Response: Kindly refer to the response given for line 99.

Line 126: provide stats for NMDA vs Basal, and also NMDA vs DHPG
Response: We have added the detailed statistics on table of Statistical analysis.

Line 131: provide stats for Dynasore vs Glut, and also Glut vs Glut+Dynasore
Response: We have added the detailed statistics on table of Statistical Analysis.

Line 137: These synaptosomes have the same level of GFAP as whole cortex lysate, therefore there is a lot of glial material in your synaptosomes. Other papers show a reduction in GFAP in synaptososme preps. For ex. see J Neurosci Methods. 2006 Nov 15; 158(1): 30-36.
Response: Kindly refer to major concern 1 put forward reviewer #1 for clarification.

Line 182: Did you measure change in the entire region shown in fig 2A? If you used a region of interest, please show the ROI.
Response: Yes, we have measured the change in the entire region and has not used any specific ROI since the entire region corresponded to distal section of the dendrite at least 50µm away from the cell soma.

Line 191: Fig 2B, is the y-axis ATP:ADP?
Response: Yes. Essentially it represents the Perceval Fluorescence ratio change over time that is corrected for the pH artifact. We have corrected the mistake.

Line 213-214: please reword for better explanation
Response: We have done it as per suggestion.

Line 206-217: Figs 2G and 2H stats appear to be comparisons with respect to response at stim onset. Please also compare DHPG vs DHPG+Aniso at their matching time points.
Response: We had done the comparison already. The statistical significance of them are represented as '#'. The details of the statistics are furnished in of Statistical Analysis.

Line 270-274: If there is labeling before stimulation, this means that there is a basal level of protein synthesis. The finding that NMDA treatement decreases the Funcat signal means that existing protein synthesis is somehow decreased, and proteins labelled during the incubation period have been destroyed. This doesn't make sense to me. Please provide an explanation for these results to explain this.
Response: We thank reviewer for this insightful comment. We do think NMDAR mediated protein degradation is the explanation for this result and we now provide some experimental evidence supporting this claim. Activity dependent degradation of proteins is known to be an important component for memory formation. The importance of protein degradation has been studied in various brain regions and various context (11) (12). Synaptic activity is known to cause degradation of various endocytic proteins, transcription factors and ribosome associated nascent peptides (13) Byraktar et. al, 2019 BioRxiv;(14). NMDA receptors are thought to activate CamKIIα responsible for redistribution of proteasomal complex and their recruitment to dendritic spine (15) Considering all these evidences, therefore it won't be surprising to assume that NMDAR stimulation can cause rapid degradation of proteins.
To test the hypothesis of NMDA dependent protein degradation, we inhibited proteasomal activity with Mg132 in cortical neurons and investigated the effect of NMDAR stimulation on FUNCAT (Supplementary Figure 2C and 2D). While NMDA led to a decrease in the FUNCAT signal, the stimulation dependent change was found to be absent in presence of Mg132 pre- treated for 1 hour.

Line 273: Please show each data point for figure 3D. Based on the error bars, the statistical significance levels appear to be unusually high. The same for figure 3F.
Response: We have included the data points in the plot as suggested by the reviewer. The details of the statistics are given in table of Statistical Analysis.

Line 314-315: Since the 1 min stimulation data is not shown, this should be removed from the protocol in fig 3A.
Response: We apologize for the mistake. We have removed it from our schematic.

Line 566-567: synaptic is presynaptic and postsynaptic compartments, but the presynaptic energy use has not been adequately addressed here. Only dendritic, so saying synaptic can be confusing here.
Response: We thank reviewer for the comment. We believe there is no scope to draw any conclusion about pre-synaptic energy level from the data presented in the manuscript. Therefore, we would be happy to rephrase to more specific 'post-synaptic'.

#Reviewer 3:

We show our gratitude to the reviewer for finding 'interesting components' in our story and for the detailed and extensive set of suggestions that has helped us in improving our study and manuscript. In the following section we have tried to address all the comments and concern from the reviewer.

"Claims include "little is known about the processes within neurons responsible for such abundant ATP outlay" which is counter to the well know processes of neuronal polarization, for example, that is well documented to be highly energetically demanding. Thus, it is very interesting that in response to a Glu stimulation, they authors can block changes in ATP by inhibiting translation. To put these results in context, however, they need to do a better job of reviewing the literature (including their ref 7). For example, they contrast their Chx findings with a block of vesicular recycling (which, according to their ref 7 is a GTP consumer rather than ATP), but don't include a control/comparison to inhibiting the processes that are thought to be the most consumptive (e.g. the Na/K ATPase that maintains ion gradients). They don't confirm that Chx or Aniso don't hit these other targets. Thus, they need to include more thorough scholarship in the introduction and perhaps s ome additional controls to put their major findings in context."

Response: We appreciate the reviewer's comment on glutamate mediated energy consumption. We have now improved the literature review on this section by including relevant references and further discussion to support why we think protein synthesis has a significant role in glutamate mediated energy consumption.

We have now also added the experimental outcomes where we investigated stimulation dependent energy consumption in presence of Na+/K+ inhibitor ouabain. We find that ouabain treatment did not have significant effect on glutamate mediated energy consumption (Supplementary Figure 1C).

We further used dynasore to inhibit dynamin, a mechanochemical protein responsible for the vesicle fission step in a GTP dependent manner. Enzymes such as Neucleoside diaphosphate Kinase is responsible for constant GTP supply which in turn is dependent on ATP levels (16). Therefore, rapid use of GTP level is expected to have reflection on the ATP level as well (Figure 1B).

Considering the effect of dynasore and ouabain we conclude that while Na+/K+ ATPase and vesicle recycling may have impact on over all neuronal energy budget, their contribution to glutamate mediated energy expenditure is not significant as compared to protein synthesis.

"ATP levels are shown to be altered and thus the authors assume this is because of changes in ATP usage. However, ATP levels are a function of both ATP consumption an ATP production. Thus, any decrease in ATP could equally be due a slow-down in ATP production rather than an increase in consumption. Indeed, ref 7 reviews literature about neuronal activity rapidly inducing mitochondrial ATP production. The authors should conduct experiments to determine whether their findings reflect changes in production, consumption, or both."

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Response: We appreciate reviewer's comment but previous reports clearly indicate that neuronal activity drives synthesis of ATP rather than inhibiting it (17)(18)(19)(20). We now have included a set of experiments where we inhibited glycolysis and mitochondrial ATP production using deoxy-glucose and oligomycin respective. We find significant contribution from both glycolysis and mitochondrial ox-phos in production of ATP in activity dependent manner which is important to robustly maintain steady state energy level on stimulation. Thus, we believe the observed effects in Figure 1B, 1C, 1H, 1I, 2E, 2G and 2H is majorly due to enhanced consumption of ATP rather than reduced production.

"The timing of the phenomena they study don't cleanly fit to their model that translation is driving loss of ATP? How do the authors reconcile the rapid inhibition of protein translation through activation of NMDA receptors which is referenced (lines 68-69, ref 16) as well as confirmed in Figure 3B seen at 2min post-stimulation with the unchanged ATP measurements following NMDA stimulation? If NMDA treatment induces an inhibition of protein translation (looks about 35% in fig 3B) and the claim is that protein translation is a major consumer of ATP within the postsynaptic density, why is there not an increase in available ATP in the NMDA-treated relative to basal conditions? If this is simply because the time points in fig 1 are too late, the experiment should be replicated at the 2 minute time point."

Response: We greatly appreciate the question by the reviewer. We believe the little impact of NMDAR mediated translation inhibition is mainly due the following reasons: 1) The extent of steady-state translation at the basal level is low inhibition of which does not lead to significant increase in the ATP level or the increase is beyond the detection limit considering the sensitivity of the assays used. 2) We also observe rapid degradation of proteins on NMDAR stimulation, which itself can contribute in some amount of ATP usage. 3) Anisomycin treatment leads to steady increase the dendritic ATP:ADP ratio with time.

All these possibilities indicate that NMDA stimulation cannot be directly approximated as protein synthesis inhibition without considering other intricacies of stimulation mediated effects.

"Key experimental details are missing from this manuscript which makes it extremely difficult to assess the veracity of statements and conclusions that the authors are making. Some of these omissions include the Mg2+ concentrations in experiments assaying NMDAR function, the timing of when protein synthesis inhibitors are added to the media, and why several of the immunoblots displayed show tubulin twice and levels of tubulin are changing between the two blots (within the same condition, same time point) ."

Response: We apologize for the confusions caused. We have used 1.25mM extracellular Mg^{2+} in the buffer in all our experiments. Only in experiments where we have tried to understand the impact of Mg2+ in the extracellular buffer, no Mg^{2+} salts were added (Supplementary figure 4G).
In all our experiments, we have pre-treated the cells with protein synthesis inhibitors for 30 minutes. We have now added an experimental timeline in the main figures to clarify the details of drug treatments.

We have loaded equal volume of proteins instead of equal amount of proteins and used tubulin levels as loading controls. We have tried to provide representative blots whenever possible for clarity.

"If all of the above work out, AMPK canonically responds to AMP levels. Therefore, the authors should also be considering the possibility that the AMPK activation is actually downstream of the ATP consumption. Are their data equally consistent with a model where ATP is consumed after Glu stimulation, and this is what then leads to elevated AMPK activity - in other words, in their summary figure should AMPK be a different position?"

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Response: The reviewer proposes an interesting explanation of AMPK function in the context of our study. However, in our experiments on synaptic activity (in both case of mGluR and NMDAR) we show an ATP independent regulation of AMPK which play a critical and non-canonical role in modulating translation. In case of mGluR, α-SNAP is thought to decouple the effect of ATP depletion on AMPK function. In case of NMDAR stimulation, Ca^{2+} dependent regulation of AMPK is thought to override the effect of ATP on AMPK function. In summary, all these results indicate that glutamate receptors employ non-canonical mechanisms to regulate AMPK function, control synaptic translation and bioenergetics.

“Line 376: the claim " indicating mGluR dependent de-novo synthesis of a-SNAP plays a critical role in AMPK dephosphorylation" is overstated. To make this claim, the need to specifically remove a-SNAP or its signaling capacity, rather than inhibiting synthesis of all proteins.”

Response: We have tried to answer in the earlier section. Our observations indicate that knocking down alpha-SNAP completely abolishes the DHPG induced dephosphorylation of AMPK in cortical neurons (DIV 15) therefore; substantiating the crucial role of alpha-SNAP on DHPG induced AMPK regulation (Figure 4I).

“Is AMPK supposed to phosphorylate eEF2 directly? This is a bit unclear in the writing in the results. It is made a bit more clear in discussion that they think this is largely indirect. Line 479: the use of the word 'direct' here implies AMPK is phosphorylating eEF2. But I don't the authors have support for that statement. They would need more biochemical evidence to establish a direct relationship than is provided here. They should perhaps rephrase more carefully.”

Response: We agree with the opinion of the reviewer. As stated in the discussion, we believe the phosphorylation of eEF2 by AMPK is not direct, rather through one or more intermediate steps in (21)between. Our experiments don’t have evidence to support direct regulation of AMPK function on eeF2 phosphorylation. Therefore, we would be happy to rephrase our conclusions.

“Line 346: The authors should introduce the direction of effect of ATP/AMP ratio on AMPK phosphorylation/activation.”

Response: we have revised our model (Figure 61H) and in that we have tried to address the concern put forward by the reviewer.

Other concerns by figure:

Figure 1:

1. “It would be helpful for the authors to add an experimental timeline, per Fig. 2F or Fig. 3A. Specifically, authors should state when the protein synthesis inhibitors were added (i.e. pretreatment or co-applied with glutamate/other agonists); and, for the cortical neuron experiments (Fig. 1A-C), clarify whether the ATP levels were measured immediately after the glutamate treatment, or after a recovery period.”

Response: We apologize for the confusion. We have now added the experimental timeline as per the reviewer’s suggestion (Figure 1A) and elsewhere it is required.
For the cortical neuron experiments (Figure 1A-C), we have lysed the cells immediately following stimulation. There was no recovery period for such experiments. Recovery period has been mentioned in the experimental timelines wherever we had such experimental designs.

2. “We would like to also see how each of the treatment conditions, and particularly protein synthesis inhibitors if they are added ahead, might change cell surface receptor expression of NMDAR, AMPAR, and mGluR within a treatment time window that matches the experimental time frame used in this and other figures.”

Response: We have done experiments according to reviewer’s suggestion. We probed for the surface mGluR and NMDAR level on anisomycin treatment for the same period of time (30 min pre-

incubation for cortical neuron experiments) as used in the original experiments. We observe an increase in the NMDAR surface level while mGluR level stayed unchanged on 30 minutes of anisomycin treatment. We have not probed AMPAR surface levels as we have only investigated NMDAR and mGluR mediated effects in our study.

3. "In the figure legend, the text, or in the Methods section, there isn't a clear explanation of why and how and why the "relative ATP level" was used for the synaptosome experiments in Fig. 1D & F, especially when a different normalization is used for cell culture experiments (ratio of ATP / Total ATP + ADP)."

Response: We apologize for the confusion. In synaptosome experiments in Figure 1H and 1I, we have done direct quantification of ATP levels using a standard curve. The ATP levels were normalized with the total protein level. The normalized ATP levels at various time points after stimulation and recovery was then expressed as a fraction of unstimulated 0 minute. For synaptoneurosomal quantification we normalized the ATP levels with total protein with the idea that with similar total protein content the adenine nucleotide pool should remain unchanged.

We shifted to measure ATP:ADP ratio in cells mainly because of two reasons:
1. The absolute levels of ATP may vary widely between cell to cell at various condition while the ATP:ADP ratio remains relatively constant over a larger dynamic range.
2. Enzymes such as Adenylate Kinase helps creating a close relationship between ATP:ADP and AMP, which in turn is sensed by various energy sensors in the cell such as AMP Kinase (Berg et. al, 2009). Therefore ATP:ADP ratio is a better approximation of available amount of energy important cellular reactions and signaling.

4. "They conclude from Figure 1 and Supplementary figure 1 that vesicle endocytosis has negligible effect on pan-neuronal ATP level (lines 130-132; mentioned again in the conclusion, lines 555-557), countering the work of Rangaraju et al 2014 (ref 16). However, this is misleading in that their experimental design does not examine presynaptic vesicle endocytosis from synaptic vesicle cycling during synaptic transmission, as application of glutamate & other receptor agonists only involves postsynaptic terminals. Therefore, while it may be that protein synthesis is a major ATP consumer in the postsynaptic neuron, it is unknown how this compares to ATP consumption in the presynaptic neuron based on the current design."

Response: We apologize again for the confusion. We agree with the reviewer that our observations don't really counter the work of Rangaraju et. al. 2014. Our observations are mostly applicable at the post-synaptic compartments. Since we have bath applied the agonists in the cultured neurons, we expect all the cells to be stimulated mostly through post-synaptic receptors. Our observations suggest that steady state ATP level of the entire neuron is not significantly affected by vesicle endocytosis on activity. Taking Rangaraju's data and our observations together indicate that there can be separate set of factors that can influence the steady state ATP level at the local compartments versus for the entire cell. We have revised the discussion on this section in our manuscript to avoid this confusion.
We also agree our experiments don't address pre-synaptic protein synthesis. Therefore, we will be happy to rephrase as per suggestion.

5. "Fig 1F -- see comment on Mg2+ block and NMDAR activation."

Response: Kindly refer to the previous comment on "Key experimental details are missing".

Figure 2:

1. "Throughout the study, the authors use NMDA to activate NMDA receptors in cultures and synaptosomes, but do not mention the concentration of Mg2+ present in media/buffer. If such solutions were used, it should be mentioned here and in the methods. If not, the effect of removing the Mg2+block on kinetics of ATP consumption and NMDAR-activation-mediated protein synthesis should be examined. (i.e. repeat experiments in Mg2+ free buffer/medium)."

Response: We have used 1.2 mM Mg^{2+} in synaptoneurosome buffer and 1mM Mg^{2+}as mentioned in the method section. We have further investigated the effect of Mg^{2+} on NMDA stimulation in cortical

synaptoneurosome by probing for Phospho/total eEF2 ratio (using buffers with and without Mg2+). The ratio was found to be increased in both cases on NMDAR stimulation and the responses in both the cases were found to be identical without any significant difference (Supplementary Figure 4G).

2. "The authors should mention when the protein synthesis inhibitor was added (pre-incubation before addition of NMDA/DHPG or simultaneous coadministration)"

Response: Protein synthesis inhibitors were always pre-treated. For cortical neurons 20 minutes of pre-treatment was done with 25μM anisomycin. For synaptoneurosomes, 10 minutes of pre-treatment was done 50μM and we have indicated that in the manuscript.

3. "Does Anisomycin alone show any effect on the ph-corrected Perceval Ratio? The authors should show these data. One can imagine that a translation inhibitor could effect an assay dependent on an overexpressed protein construct."

Response: The effect of anisomycin treatment has been mentioned in supplementary figure 2C. Anisomycin did cause an increase in the pH corrected Perceval fluorescence over time. We did not observe any significant difference in Perceval expression level over 40 minutes incubation period (20 minutes pre-treatment + 20 minutes imaging). Since Perceval can faithfully report the cellular ATP:ADP ratio change over a ~10 fold range of change in sensor expression (Tantama et. al, 2013), we believe the anisomycin mediated change in PercevalHR fluorescence is due to inhibition of global protein synthesis at baseline.

4. "The change in ATP/ADP ratio in unstimulated and anisomycin-only-treated conditions should be moved to the main figure (2G and 2H) from Supplementary Figure 2, and included in the statistical analysis of Fig 2C-E."

Response: We thank reviewer for the suggestion. We have now moved the following traces to the main figure. We have also included the basal group for the analysis of previous Figure 2C-2E (currently Supplementary figure 2C-2E). We have shown the effect of anisomycin compared to untreated control and have quantified the effect of anisomycin over time on a separate analysis.

5. "Correct figure 2E legend to "10 minutes" from "2 minutes."

Response: We apologize for the mistake. Correction has been made.

6. "Figure 2B seems to call for a repeated measures ANOVA"

Response: We thank reviewer for the suggestion. Considering the range of variance being different for each of the groups, the ANOVA would hardly find any significance. Therefore, while in the line graph we tried to represent temporal kinetics, statistical comparison has been done on specific time points in separate analysis (Supplementary Figure 2C-2E).

Figure 3

1. "In Figure 2, it was suggested that activation of NMDAR induces delayed protein synthesis (5-10 minutes). The results from Fig 3A-D suggest that NMDAR activation induces a rapid inhibition of translation. It would be interesting to see whether there is de novo protein synthesis after 10 minutes of NMDA stimulation."

Response: It is indeed an interesting question that we want to pursue. We have now completed three set of experiments to investigate the effect of 20 minutes after 20μM of NMDA addition on cultured neurons. We observed significant increase in the FUNCAT signal which corroborated our hypothesis that indeed NMDAR stimulation activates translation following its rapid inhibition (Supplementary figure 3C and 3D).

2. "Data in Fig 3D could be annotated better (No explanation of B2, D2, N2, etc in figure legend or text) or be removed altogether, as it is the same information as 3C."

Response: We thank the reviewer. We have now shifted the analysis to the supplementary figure 3B.

3. "In Fig 3B and E, add a zoomed-in inset of images at the dendrite to visualize protein synthesis/eEF2 phosphorylation at the dendritic level."

Response: We have acquired the images such that we could quantify the FUNCAT signal from the entire cell rather than any specific dendrite. Since, we don't have a corresponding high-resolution image of the dendrites of the specified cells, we avoided any representative dendrites in the manuscript figures.

4. "3B-3C and 3E-3F, graphed data is normalized to Map2 intensity. Please show these Map2 images in the main figure adjacent to the peEF2 images."

Response: We have provided the MAP2 images as per the suggestion in all the figures.

5. "Fig 3H: further clarify how the relative eEF2 phosphorylation levels were quantified here ((p-eEF2/ Tubulin) / (initial p-eEF2 / initial tubulin)?"

Response: We apologize about the confusion around the method. We have run the same samples in two different gels and did western blotting of both. In one of them we probed for phospho proteins viz phospho-eEF2 and tubulin for each lane. The phospho-eEF2 values for each lane was normalized to tubulin band intensity. In the other blot we repeated the same for total-eEF2. Once we obtained the normalized values of both phospho and total eEF2, we made a ratio of these normalized values to obtain phospho to total ratio for each lane. Therefore, once we obtained phospho to total eEF2 values for every time point, the other time points were represented as fraction of the initial time point which is 0 minute unstimulated condition in this case.

6. "Fig 3H should also include the trace for the unstimulated condition (Supplementary figure 4B), include discussion of the increase in phosphorylation seen in the unstimulated condition over time. The statistical analysis done here should include the unstimulated condition (also increase sample size for unstimulated? "n {greater than or equal to} 2 animals per group")"

Response: We increased the sample size to minimum of 3 for each protein of eEF2, AMPK and α-SNAP in their unstimulated conditions over all the time points (Supplementary Figure 4A). We did not find significant difference in their phosphorylation level over time under basal condition. This result justifies our method to keep the baseline phosphorylation in the separate analysis. In this way we feel, Fig 3H helps to appreciating the difference in eEF2 phosphorylation response between the two receptors better.

7. "Fig 3H It's not clear why two Tubulin blots are shown for each stimulation set. Furthermore, at some time points (DHPG @ 2 mins, 5 mins), Tubulin levels are changing relative to each other more so than p-eEF2/T-eEF2 levels."

Response: We apologize for the confusion. The two tubulin blots were used to normalize two version of the proteins i.e. phospho and total forms of eEF2 or AMPK. Kindly refer to Figure 3 Q5 for further details.

We have loaded equal volume of sample instead of equal amount of protein in each lane which is likely the reason for variation in tubulin levels and could also be a bias of the representative. However, we have verified our observations over multiple animals and the values have been verified statistically to reach relevant conclusions.

Figure 4

1. "A better representative sample image for DHPG + Aniso in Fig 4C is needed where the lanes are not overlapping."

Response: We have made the changes as per the suggestion of the reviewer (Figure 4C).

2. "Fig 4D & F should include traces from Unstimulated & Unstimulated + Aniso to show the effects of protein synthesis inhibition on AMPK phosphorylation & alpha-SNAP synthesis."

Response: We have added the phospho to total ratio of eEF2, AMPK and α-SNAP levels on unstimulated basal condition in presence or absence of anisomycin in supplementary figure (Supplementary figure 4A and 4B). Kindly refer back to Figure 3 Q6 for further information.

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3. "Clarify which condition the images in Fig 4G is from, and include representative images from both basal and DHPG conditions + some zoomed in images."

Response: The representative image of colocalization analysis has been shown from DHPG stimulated cells. As per the suggestion by the reviewer we have now shown the colocalization in both basal and DHPG stimulated conditions with some zoomed in images added (Figure 4G).

4. "4A-4B, graphed data is normalized to Map2 intensity. Please show these Map2 images in the main figure adjacent to the peEF2 images because the values shown in the graph suggest that the chosen images have vastly different Map2 expression levels, which probably indicates they are not faithful representative images."

Response: We appreciate the reviewer's concern and now we have altered the images as per suggestion. We also have now added the MAP 2 images in the main figure.

5. "Figure 4H - correlation needs to be calculated w/in the cell. Not the whole image. Otherwise the finding might just reflect the difference between the inside and outside of the cell. More importantly, this kind of imaging is supportive that the proteins could be interacting, but does not have the resolution to say they are associated, as suggested on line 378. True molecular interactions would need to be shown by Co-IP or Fret, or other appropriate assay."

Response: We have re-analyzed our data as per suggestion. The correlations were calculated within the area of the cell. We first created a cell mask using the intensity values from one of the channels (α-SNAP) from the cell and the correlation was further calculated within the area of the mask.
Though we could not do co-IP experiments due antibody related issues, we have provided evidences of α-SNAP mediated regulation by alternate methods (Figure 4I). We agree to the reviewer that colocalization can't conclusively provide evidence of association due to the resolution limit of light microscopy. Therefore, we have rephrased the association between SNAP and AMPK as 'colocalization'.

Figure 5

1. "5A: again, not clear why two tubulin blots are shown and why they are discordant."

Response: Kindly refer to Figure 3 Q7 for clarification.

2. "5C-E: Need some sort of color legend/scale."

Response: We apologize for this omission. Now we have added the scale to the figure.

3. 5C-5G: Is this novel? That NMDA receptor activation leads to rapid calcium influx has been known for quite some time; not only that, the calcium imaging experiment doesn't directly tie NMDAR-mediated rapid calcium influx to AMPK activation. Should be supplemental info if authors are just showing that they can induce calcium influx in neurons in response to NMDA in their hands.

Response: We appreciate reviewer's question and agree this data 'per se' is not novel. The major point of the experiments done in figure 5C-G is to corroborate the idea that just NMDA addition can lead to Ca^{2+} entry inside the cell. This helps other concerns such as in presence of Mg^{2+} we could successfully observe the rapid entry of Ca^{2+}. The following are the reasons of importance which justify our inclusion of this data:

- This result confirms that, NMDA addition cause receptor activation in the conditions we have used (in presence of Mg2+ -1.25mMor in absence of glycine).
- NMDA induced intracellular Ca2+ continued to stay higher than baseline as long as stimulant was present. This essentially emulates repeated burst of Ca^{2+} signals required for CamKKIIB activation as suggested by (Ref?) .
- We also successfully show that the signal is majorly contributed by extracellular Ca2+ as opposed to other ideas such Ca2+ released from intracellular stores.

4. Methods section states that Fmax was calculated with 10uM ionomycin and 2.5 mM Ca++. The figure states 10 mM Ca; authors should clarify which condition was used for Fmax calculation.

Response: We again apologize for the mistake made in the methods section. Fmax was calculated in presence of 10mM Ca^{2+} with 10µM ionomycin and now we have corrected this in the manuscript

5. "Figure legend for 5G should state the time point for when ΔF/F0 was measured (i.e. 5 minutes into treatment, average of 30 sec)."

Response: ΔF was calculated maximum fluorescence after NMDA addition – Fluorescence before NMDA addition ($F^{max}_{NMDA} – F_0$). It wasn't at a specific time point. Rather F^{max}_{NMDA} value was obtained from any of the time points after NMDA addition but before ionomycin addition which showed the maximum Fluo-8 fluorescence value. ΔF was further divided by F0 to obtain stimulation mediated change in fluorescence. To avoid the confusion, we would rename the Y axis of figure 5G as "peak ΔF/F0".

Figure 6

1. "Map2B stain in Supplementary figures should be shown with the representative images in 6A, C, E, G."

Response: We have modified the arrangement of figures as per the suggestion.

2. "So here, the authors state that the AICAR and CC were applied for a full hour before receptor agonists were added (5min). It is difficult to tell whether this was also the case for the other treatments from previous experiments. Again, adding a timeline of treatment here would be helpful."

Response: We thank the reviewer for this comment. Now we have added the experimental timeline wherever they are required as per the suggestions.

We thank all the reviewers for their constructive criticism and valuable suggestions. We certainly feel the manuscript is significantly improved and strengthened because of these comments and now we are confident that we have addressed all their concerns also.

References:

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2nd Editorial Decision 24 October 2019

Thank you for the submission of your revised manuscript. We have now received the enclosed comments from the referees as well as cross-comments.

As you will see, while referees 1 and 3 are more positive, referee 2, an expert in electrophysiology, is not convinced by the current set of data, and referee 1 agrees with the remaining concerns. It is therefore essential that all experiments based on neuronal cell cultures and that relate to the conclusion that a substantial fraction of neuronal energy is used for global the de-novo translation in the post-synapse are repeated on proven-to-be-healthy neuronal cultures (this concerns figures 1 and 2, may be figure 3, and the relevant suppl figures). I am aware that this means a second round of major revisions, but in the absence of conclusive data, we cannot offer to publish your manuscript. All other referee concerns as listed below will also need to be successfully addressed.

Referee 2 further remarks in her/his cross-comments (see below) that her/his previous concerns were addressed in a sub-optimal manner, and I certainly agree that all data need to be discussed in an unbiased manner, and that all referee concerns need to be thoroughly addressed, which was not the case in the last round of revision.

Taken together, this manuscript is still a borderline case. I am willing to consider a newly and significantly revised study, but we can only offer to publish it if all concerns can be successfully and carefully addressed.

Alternatively, I could share your revised study and the referee reports with Andrea Leibfried, executive editor at Life Science Alliance. Less demanding revisions might potentially be required for LSA, but the exact requirements will need to be discussed.

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Please let me know how you prefer to proceed.

REFEREE REPORTS

Referee #1:

In their revised manuscript, the authors have included a number of new experiments and analyses that greatly improve the quality and add necessary stringency to their study. The have sufficiently answered the points raised in my original review. The findings of this study remain surprising in that previous work on energy consumption in neurons has stressed the dominant demand of membrane polarization and vesicle recycling. In the revised manuscript the authors have done a better job to frame their results in the context of the existing literature. Overall, the current study has been carefully conducted and will be an interesting contribution to the field. I would suggest that the authors consider the following suggestions:
They should include a justification for changing the ATP quantification method between cortical cultures and synaptoneurosomes from the ATP/(ADP+ATP) ratio to ATP/total protein. This is especially important given that they are using protein synthesis inhibitors, that feasibly could reduce the total protein level.
Figure S4 needs to be labeled, and ideally the western blot showing reduced a-SNAP levels should not be spliced.
The manuscript needs thorough copy editing (esp. spelling, including in figures).

Referee #2:

While your group has now shown that your cultures can fire APs and that the neurons are forming release competent synaptic connections, measured as a relatively low level of action potential independent vesicle release, you have not shown that your cultures have normal levels of spontaneous action potential activity that can trigger neurotransmitter release. I stated the in my original review "...it needs to be determined that synaptic transmission occurs in the cultured neurons, and that transmitter release can follow physiological firing frequencies." Synaptic transmission needs to be shown, and transmitter release needs to be shown to occur at physiological firing (AP) frequencies. There are a number of papers where spontaneous action potential induced neurotransmitter responses are shown. For example, in reference 28 (Sun, et al) from your manuscript, the cultured neurons at DIV14 show spontaneous action potential activity at a frequency of 10 Hz. This is a good indication that the neurons they used are firing in a network type fashion. This has not been shown for the neurons that were used for you experiments.

It's also worth noting several other differences between the neurons used in your experiments and those used in reference 28 (Sun, et al). First, for ref 28, the average resting membrane potential is -60 mV, which basically indicates the neurons in their culture are healthy. The average resting potential for neurons in your experiments was almost 10 mV higher (51 mV, line 151). In their neurons, the action potential amplitude was at 97 mV (overshoot minus the negative membrane potential in current clamp) while your neurons had an AP amplitude of 72 mV. The overshoot of the AP was 27mV for their neurons which indicates a robust AP, compared to 12mV overshoot in your neurons. It's important to note that even a small reduction in the peak amplitude of the AP will cause a large decrease in the amount of neurotransmitter release. In addition, in your neuronal cultures, the spontaneous release rate was less than 2 Hz, compared to just under 10 Hz for ref. 28 (fig 3d). Finally, the top trace you show for spontaneous activity in the presence of TTX (fig 1E) is quite noisy, which in my experience tends to happen when the neurons aren't in their best condition.

In summary, you have not shown that neurons in your cultures are firing action potentials on their own. Therefore, it's completely possible, perhaps likely, that the neurons in your culture are "quiet" and therefore experience little to no action potential induced synaptic transmission. You have not ruled out that your cultures are effectively in a pathology state with little or no action potential generated synaptic communication. You need to show that the neurons in your cultures fire APs at a reasonable frequency on their own, and these APs release neurotransmitter. Most importantly, this needs to be shown for the same batch of neurons that you do a new round of ATP consumption,

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metabolism (energy use) experiments on. This applies to all of the experiments done with cell culture. The dendritic vs soma differences in ATP consumption are an interesting result, but they need to be done neurons that have good levels of spontaneous AP and AP stimulated release. The synaptosome experiments (Fig 1F-I) were not done in culture, and the results clearly show an mGluR mediated reduction in ATP levels that is not seen for NMDA. However, it's also important to note that synaptosomes are also devoid of AP activity and AP stimulated transmitter release. Therefore, showing spontaneous AP's and AP generated neurotransmitter release is important to explain your findings on energy use by protein synthesis.

I have an additional concern or confusion in comparing figure 2C with 3C. If protein synthesis is the major reason for energy consumption, and the activity dependent increase in energy use is higher in the dendrites than in the soma (Fig 2C) then it's difficult to understand the FUNCAT experiments because there is a noticeable increase in both the soma and in the dendrites (Fig 3C).
How do these two findings relate to each other concerning protein synthesis and energy use in the dendrites vs soma?

For the rest of the manuscript, I think that the findings for mGluR vs NMDA effects on translation are very interesting. It was good to test the effects of inhibiting protein degradation with respect to the decreased FUNCAT signal caused by NMDA stimulation. The FUNCAT experiments are convincing, and the connection with phosphorylated Eukaryotic Elongation Factor 2 (p-eEF2) works well. The AMPK reduction in activity by activation of mGluR is unexpected (line 442), which appears to require translation, and indicates that AMPK's activity is highly regulated and complicated. The increase of alpha-SNAP levels by mGluR activation demonstrates this, and other AMPK specific phosphatases are likely to also affect AMPK activity. Finally, the importance of AMPK in the mGluR and NMDAR effects on translation was shown by pharmacologically activating and inhibiting AMPK activity.

While it's also possible that regular glutamate release through spontaneous AP activity could cause changes in the mGluR and NMDA effects on translation, I think that this would be a lot of work to fully understand. My concern for the current manuscript is primarily the potential problem of low levels of neuronal activity causing a condition where acute stimulation with glutamate causes an larger than normal increase in translation levels, with lower than normal levels of electrical activity in the neurons.

Additional concerns by line or fig. number:
Line 106-7: the comparisons in fig 1B are between Glu vs Glu + translation inhibitors. Please compare Glu+translation inhibitors to baseline if you are going to say that the translation inhibitors completely offset the effect of glutamate.
Line 118: The MPEP treatment has not compared to baseline, and the anova info table is not helpful as presented.
Line 127: NMDA is only 3 data points
250-2: Dendritic energy use increases with DHPG treatment, but somatic energy use is not affected. What is the area ratio for the soma vs. dendrites in these neurons, and how does that work with the results from figure 1 for lysates that include neuronal soma, dendrites, and axons plus of course glia?
Line 334, 339, and anywhere else: change word: "till"

Referee #3:

I believe the authors scientifically addressed the majority of my raised concerns, as well as those of other authors where i had sufficient expertise to evaluate them, and thus scientifically it is probably now suitable. However, the manuscript still suffers from an excessive amount of typos and grammatical errors. Here are some e.g.'s of typos and writing that could be clarified on first page:
Line 45: "The brain"
Line 46: "This consumption"
Line 47, 59: Typo: I don't think 'evidences' is used as a plural noun.
Line 50: "Lead" (the relevant noun here is 'Conditions' so the verb is lead, rather than 'leads')
Line 85: "How is activity"
Line 88: "a large amount"

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Line 37,55 and throughout: Use of "after" or "upon" rather than "on" here and throughout the manuscript might be a more clear word choice.
Line 94: "after both stimuli"
Line 95: rephrase
Fig 1A: period? Preload?

After Line 95, I've stopped with this kind of editing and focused on scientific review, but the manuscript continues to need deep editing for standard English usage before it will be suitable for publication. However, this is probably more a role for a copy editor than a scientific reviewer. Scientifically I think they addressed my concerns. I would note that in addition to copy editing, it would not hurt to think about the order and framing of the newly added data a bit. For example the electro-physiological analysis added for one of the reviewers to show the neurons are healthy? For a naive reader not familiar with what the reviewers asked for, it is not really explained why it is there. Perhaps, as it is mostly a control, it might be more of a method section thing anyway.

one last typo - Line 468: "the complex a regulation" - not sure what this is meant to say.

Cross-comments by referee 1:

I fully agree with reviewer #2; the demonstration of normal AP firing in these cultures is important to rule out that the presented (surprising) results are a consequence of unhealthy neurons/suboptimal culturing conditions. As I am not an expert on electrophysiology, I have reviewed the manuscript mainly from a protein synthesis perspective, and I would refer for the e-phys details to reviewer #2's judgement. Following reviewer #2's argument, the neurons are probably not very healthy and the results concerning energy consumption by protein translation might be caused by the state of the neurons. The problem I see here is that even if the authors would now show that they can achieve the values referenced in reviewer #2's critique, all other experiments have already been performed in cultures of doubtful health. Therefore, any 'revision' would be more like a total do-over of the paper. These issues affect primarily the claim of energy use by translation, while the mGluR vs NMDA effects on translation are less likely to be affected by the health of the cultures.

Cross-comments by referee 2:

I agree with reviewer number 1, that the health of the culture primarily affects the results for the ATP consumption experiments done in cell culture in figures 1 and 2 (and the relevant suppl figures). It could also affect the FUNCAT experiments (Fig 3), but I agree that the specific effects of mGluRs vs NMDARs and the pathways are less likely to be affected. I know this is a lot of work, but it's difficult for me to give the authors the benefit of the doubt, given how they've presented the data so far (see below).

I also want to emphasize that the way in which the data continue to be presented, and their responses to my concerns are elevating my concerns. As one example, in lines 106 to 107 they say "This stimulation dependent reduction was absent in presence of protein synthesis inhibitors anisomycin (25µM) or cycloheximide (350µM) (Figure 1B)." But there is a reduction compared to baseline, I ran the numbers based on their scatterplot. For figure 1b, I get glut =0.63 (values: 1.05,1.05,1.03,1.01,0.86) and glu + aniso is 0.87 (values: 0.92, 0.92, 0.88, 0.85, 0.8). There is a big reduction but not complete elimination with protein synth inhibitors, so why not just say that? I understand the problems with doing multiple t-tests, but for what it's worth, the difference between control and glu+aniso is significant at p= 0.018. Something else is using energy, which actually makes sense, so their reluctance to acknowledge this, just as one of many examples, concerns me. Rather than do the statistical comparisons that were requested, they skipped direct comparisons that were asked for, and maintained direct comparisons that were not the best ones to

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do, but appear to have been chosen because of the presence or absence of statistical significance.

Another example is the concern I had from the first review of the paper, which is that "protein synthesis [could be] necessary to initiate or maintain another process that consumes energy, such as transporter activity, vesicle release + recycling, etc." I then suggested the use of a non-canonical amino acid that would allow protein synthesis but prevent the production of functional proteins. They dismissed my suggestion with three arguments. The first one was that non-canonical aa's don't affect protein function or location, and this isn't correct because there are nc aa's that inactivate protein functions (see Essays Biochem. 2019 Jul 3; 63(2): 237-266.,Using genetically incorporated unnatural amino acids to control protein functions in mammalian cells). Next they said the nc aa's would compromise the animals health, but they are using cell lines and the nc aa's aren't there for days, although since their cells aren't healthy enough to fire spontaneous APs, perhaps the brief incubation with nc aa's would have damaged the cells further. Finally, they said that nc aa's would bias energy utilization to break down the inactive proteins. This makes sense, but it's not clear to me how much protein synthesis was occurring before glu stimulation and if it's enough to cause significant build up of proteins before glu stimulation. *Lastly, they said "We will incorporate the possibility raised by the reviewer in the discussion which will no doubt enhance the interpretation of the paper", but they don't provide line numbers, and I do not see this point addressed anywhere in the discussion section. I've also looked through the results and can't find it there either. Because of this, it feels like the authors ignore any alternate explanation of their work, and they resist showing comparisons that don't completely support their hypothesis and this concerns me. *

In summary, I agree with reviewer 1, that the relevant experiments need to be repeated with cells that are proven to be healthy for the same batches on the same day as each experiment is run.

2nd Revision - authors' response 29 January 2020

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Response to reviewer's comment

We thank the reviewers for their useful suggestions and critical analysis of our work. We have carefully gone through their comments, discussed their point of view with the authors and other experts and have tried to explain their queries.

Concerns Related to Reviewer 1:

"In their revised manuscript, the authors have included a number of new experiments and analyses that greatly improve the quality and add necessary stringency to their study. They have sufficiently answered the points raised in my original review. The findings of this study remain surprising in that previous work on energy consumption in neurons has stressed the dominant demand for membrane polarization and vesicle recycling. In the revised manuscript the authors have done a better job to frame their results in the context of the existing literature. Overall, the current study has been carefully conducted and will be an interesting contribution to the field. I would suggest that the authors consider the following suggestions: They should include a justification for changing the ATP quantification method between cortical cultures and synaptoneurosomes from the ATP/ (ADP+ATP) ratio to ATP/total protein. This is especially important given that they are using protein synthesis inhibitors, that feasibly could reduce the total protein level."

Response: We thank the reviewer for finding our revised manuscript of 'greatly improved quality' and having the 'necessary stringency' to make an 'interesting contribution to the field'.

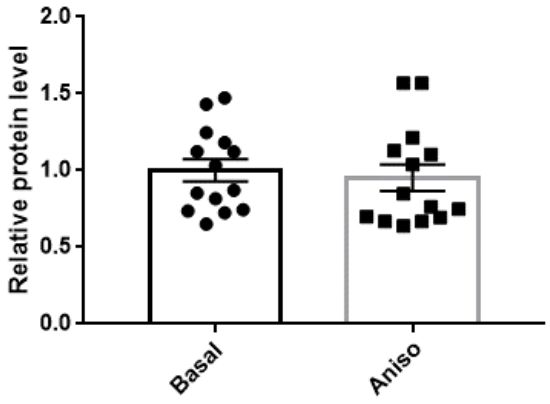


Figure 1: Comparison of protein content between control and anisomycin treated samples. Data presented below from BCA absorbance values; n=14 animals, statistical significance could not be found with unpaired Student's t Test

Following is the justification for changing the ATP quantification method between neuronal cultures and synaptoneurosomes. The total protein was quantified to collect an equal amount of synaptoneurosomes which unlike the neurons could not be counted before plating. The total protein was used to normalize with the assumption that a relatively constant adenine nucleotide pool will exist in a certain amount of protein which has been conventionally used in the literature to normalize ATP levels (1) (2). In our experiments, we did not observe any significant difference in the total protein level between control and anisomycin treated samples verified by BCA based method (Figure 1). Considering the robust stability of the synaptic proteins (3)(4) along with our BCA measurements, it appears that anisomycin treatment for such a short period does not alter the total protein level. This shows that our observations follow similar trends with two

independent approaches. However, we have added an explanation in line no 148-149 to clarify this.

"Figure S4 needs to be labeled, and ideally the western blot showing reduced a-SNAP levels should not be spliced. The manuscript needs thorough copy editing (esp. spelling, including in figures)."

Response: We apologize for this. We have added a new representative blot with both lanes together.

We apologize for all the copy editing and labeling related issues. We have now taken the utmost care to correct the typographical and grammatical errors as suggested.

Concerns Related to Reviewer 2:

"While your group has now shown that your cultures can fire APs and that the neurons are forming release competent synaptic connections, measured as a relatively low level of action potential independent vesicle release, you have not shown that your cultures have normal levels of spontaneous action potential activity that can trigger neurotransmitter release. I stated in my original review "...it needs to be determined that synaptic transmission occurs in the cultured neurons, and that transmitter release can follow physiological firing frequencies." Synaptic transmission needs to be shown, and transmitter release needs to be shown to occur at physiological firing (AP) frequencies. There are a number of papers where spontaneous action potential induced neurotransmitter responses are shown. For example, in reference 28 (Sun, et al) from your manuscript, the cultured neurons at DIV14 show spontaneous action potential activity at a frequency of 10 Hz. This is a good indication that the neurons they used are firing in a network type fashion. This has not been shown for the neurons that type-dependent for your experiments.

It's also worth noting several other differences between the neurons used in your experiments and those used in reference 28 (Sun, et al.). First, for ref 28, the average resting membrane potential is -60 mV, which basically indicates the neurons in their culture are healthy. The average resting potential for neurons in your experiments was almost 10 mV higher (51 mV, line 151). In their neurons, the action potential amplitude was at 97 mV (overshoot minus the negative membrane potential in current clamp) while your neurons had an AP amplitude of 72 mV. The overshoot of the AP was 27mV for their neurons which indicates a robust AP, compared to 12mV overshoot in your neurons. It's important to note that even a small reduction in the peak amplitude of the AP will cause a large decrease in the amount of neurotransmitter release. In addition, in your neuronal cultures, the spontaneous release rate was less than 2 Hz, compared to just under 10 Hz for ref. 28 (fig 3d). Finally, the top trace you show for spontaneous activity in the presence of TTX (fig 1E) is quite noisy, which in my experience tends to happen when the neurons aren't in their best condition.

In summary, you have not shown that neurons in your cultures are firing action potentials on their own. Therefore, it's completely possible, perhaps likely, that the neurons in your culture are "quiet" and therefore experience little to no action potential induced synaptic transmission. You have not ruled out that your cultures are effectively in a pathology state with little or no action potential generated synaptic communication. You need to show that the neurons in your cultures fire APs at a reasonable frequency on their own, and these APs release neurotransmitters. Most importantly, this needs to be shown for the same batch of neurons that you do a new round of ATP consumption, metabolism (energy use) experiments on. This applies to all of the experiments done with cell culture. The dendritic vs soma differences in ATP consumption is an interesting result, but they need to be done neurons that have good levels of spontaneous AP and AP stimulated release. The synaptosome experiments (Fig 1F-I) were not done in culture, and the results clearly show a mGluR mediated reduction in ATP levels that are not seen for NMDA. However, it's also important to note that synaptosomes are also devoid of AP activity and AP stimulated transmitter release. Therefore, showing spontaneous AP's and AP generated neurotransmitter release is important to explain your findings on energy use by protein synthesis."

Response: In the initial comments, the reviewer expressed concerns regarding the "low" baseline synaptic activity and stated: 'My minimum recommendation to address the concerns outlined above is that the action potential kinetics (shape and

amplitude), threshold, and firing frequency, all need to be measured for the cultured neurons and the values need to be in the range of physiological levels. In addition, it needs to be determined that synaptic transmission occurs in the cultured neurons, and that transmitter release can follow physiological firing frequencies.'

Accordingly, we evoked the action potentials (APs) by employing multiple current steps and measured the properties of APs as recommended (Manuscript Figure 1D and S1H). We also recorded spontaneous miniature post-synaptic currents (mEPSCs) and measured their frequency and amplitude (Manuscript Figure 1E and S1K). These parameters were comparable to the published values from rat neurons signifying that our culture displays spontaneous synaptic transmission. We realized that "within physiological range" indeed represents a range of values reported by various publications that we have presented in Table 1 below.

Table 1:

Parameter	Low-density value ($2*10^4/Cm^2$)	High-density Value ($4*10^4/Cm^2$)	Published value
Resting Membrane Potential	-51.25 ± 2.04 mV	-57.68±1.381 mV	-57 ± 2 mV (5) -56 ± 2.2 mV (6) -50 and above (7)
Capacitance	47.8 ± 6.48 pF	59.95±4.844 pF	Median 40 pF (8)
Input Resistance	353.4 ± 29.23 MΩ	184.6 ± 24.54 MΩ	168 ± 23 MΩ (5)
Action Potential Amp	72.76 ± 3.464 mV	88.69 ± 3.828 mV	63.9 ± 1.1 mV (6) 78 ± 2 mV (9)
Action Potential Spike Half width	2.326 ± 0.266 ms	1.862 ± 0.1392 ms	1-2ms (7)
Action potential Threshold	-40.45 ± 1.76 mV	-46.81 ± 2.28 mV	-40 (6)
Burst Frequency	Not recorded	15.55 ± 1.18/min	<5 /min recorded with MEA (10)
Burst Duration	Not recorded	1.577 ± 0.111 s	<1 s recorded with MEA (10)
mEPSC Amp	-35.5 ± 5.03 pA	-70.13 ± 10.26 pA	Median <15pA (8)
mEPSC Freq	1.845 ± 0.42 Hz	6.368 ± 0.866 Hz	Median 2 Hz (8)

With the new batch of cultures, we repeated glutamate stimulation in the presence or absence of anisomycin. Anisomycin significantly rescued the glutamate-mediated dip in the ATP level as we observed in figure 1 of the manuscript.

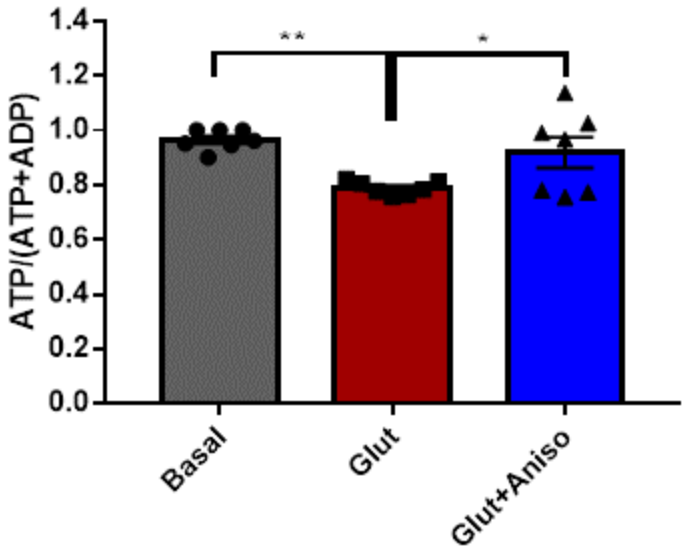


Figure 2: Bar Graph representing neuronal ATP/ADP ratio on glutamate stimulation in the presence and absence of anisomycin.; n=7 wells from 3 independent platings, statistical significance was tested with one-way ANOVA followed by Bonferroni's multiple comparison test. p= 0.0051 between basal and glutamate and p= 0.0436 between glut and glut+aniso. Difference between Basal and glut+aniso was non-significant.

Based on the previous reports, we also argue that the neuronal connections formed in the culture dishes are highly stochastic and non-stereotypic. Therefore, many of the electrophysiological measurements from neuronal cultures show a large variance that needs to be considered while comparing the central tendencies (such as average) of two populations.

Here, we discuss elaborately the problems of relying on a single reference in evaluating our data:

1. Sun et al., 2018, for example, used embryonic mouse cortical neurons while we have used rat cortical neurons. A large body of evidence suggests that significant morphological and electrophysiological differences exist between the rat and the mouse neurons (11)(12). Therefore, the species-specific aspects can give rise to the significant differences observed between values reported by Sun et. al, 2018 and our manuscript. Hence while we cited this paper in our manuscript, Sun et. al, 2018 isn't the ideal reference to directly compare the values in this case.

2. The reviewer's following comments "the cultured neurons at DIV14 show spontaneous action potential activity at a frequency of 10 Hz" and "You need to show that the neurons in your cultures fire APs at a reasonable frequency on their own, and these APs release neurotransmitters" is again based entirely on Sun et. Al, 2018. However, Sun et. al, 2018, measured the frequency of sEPSCs that is known to be influenced by both action-potential dependent and independent current components (13)(14)(15). Therefore, the expected frequency of spontaneous AP frequency of 10 Hz is not well-founded.

3. The connectivity and therefore the spontaneous burst frequency of neurons in dissociated cultures vary with plating density (16)(17). Cells in low-density cultures develop a lesser number of synapses compared to high-density

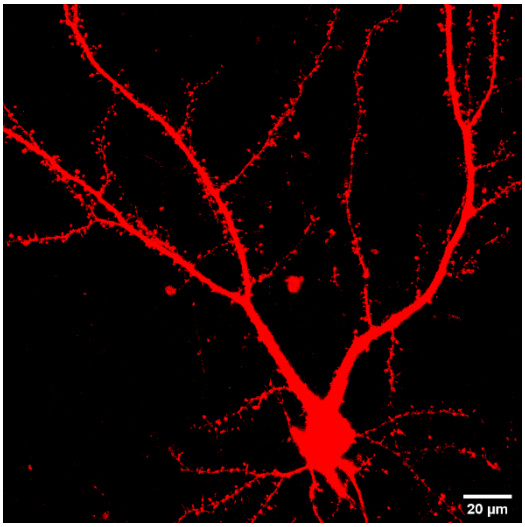
cultures. Hence many times, healthy low-density neurons can't be induced to burst (18). In the first version of the revised manuscript, we recorded from neurons at a lower density ($1.8X10^4$ cells/cm^2) originally used for imaging-based experiments in the first version of the manuscript [mentioned in the **Cell Line and Primary Neuronal Culture part** of the materials and methods section]. This density was lower compared to the reported high-density neuronal cultures used for recording spontaneous action potential (16)(17). Hence, we decided to record from a relatively higher density of neurons ($4X10^4$ cells/mm^2) that we have used for the ATP measurement and other biochemical assays previously. The culture displayed bursts of spontaneous action potentials which we did not record last time. We also observed a robust increase in the mEPSC frequency and amplitude with higher density suggesting that an increased number of connections have formed in this culture.

Here we also present a list of the experiments coupled to the relevant neuronal plating density used for those experiments.

Table 2:

Assays in the Manuscript with Primary Neuronal Culture	**Density Used**
ATP:ADP Ratio (Biochemical)	$4X10^4$ /Cm^2
ATP:ADP (Imaging)	$2X10^4$ /Cm^2
Western Blotting of eEF2	$4X10^4$ /Cm^2
FUNCAT and Immunolabeling	$2X10^4$ /Cm^2
Ca^{2+} Assay (Imaging)	$2X10^4$ /Cm^2
KD of α-SNAP and Western Blotting	$4X10^4$ /Cm^2

Our observations clearly demonstrate that the density of neurons did not alter the outcome of the experiments (the link between ATP consumption and activity mediated protein synthesis) while they do show different electrical properties as reported earlier. Thus, the extent of ATP consumption in response to glutamate stimulation appears to be cell-autonomous and independent of the spontaneous firing properties of these neurons. Hence, the extent of activity mediated protein synthesis as verified by multiple studies (19)(20)(21) is not "more than normal".



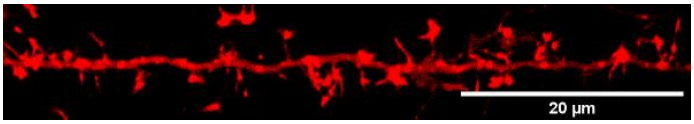


Figure 3: A representative neuron showing profuse arborization and presence of dendritic spines in various stages of maturation at DIV 15 plated at a density of $4X10^4$ cells/cm^2. Neurons were labeled with Lifeact-mcherry overexpression to visualize the dendritic spines.

4. We agree with the reviewer that we don't have enough evidence to support the claim that 'protein synthesis inhibitors completely offset the glutamate-mediated energy consumption'. Our data suggest that about 60-65% of the consumed energy can be recovered by anisomycin preincubation. The rest 35-40% can be attributed to various other processes that accompany synaptic activation like ionic rebalancing, endocytosis, organellar movement, cytoskeletal rearrangement, autophagy, global protein degradation, and others. We have now discussed these issues in line no. 628-631 of our manuscript for clarification.

In summary, we present the data that convincingly show that the neuronal cultures used in the manuscript are capable of firing spontaneous bursts of action potentials. The 'relatively lower' values measured for synaptic transmission was due to sparse connectivity and not due to any cell health issues.

I have an additional concern or confusion in comparing figure 2C with 3C. If protein synthesis is the major reason for energy consumption, and the activity-dependent increase in energy use is higher in the dendrites than in the soma (Fig 2C) then it's difficult to understand the FUNCAT experiments because there is a noticeable increase in both the soma and in the dendrites (Fig 3C).

How do these two findings relate to each other concerning protein synthesis and energy use in the dendrites vs soma?

Response: We agree with the reviewer that the absence of energy consumption in the soma in-spite of an increase in FUNCAT intensity is a surprising and interesting observation. Although we don't have direct evidence to explain the observation, our reasoning is as follows:

1. While there is an increase in the FUNCAT signal in both soma and dendrite, the subsequent impact of protein synthesis on energy levels depends on the amount of burden handled by energy supply pathways locally within various neuronal compartments. The heterogeneous distribution of other potential energy sinks such as Na+/K+ ATPase or vesicle endocytic machinery (22)(23), heterogeneity in the distribution of mitochondria and glycolytic enzymes (24) and their distinct regulation of function locally (25) can create a spatially variable impact over neuronal energy level.

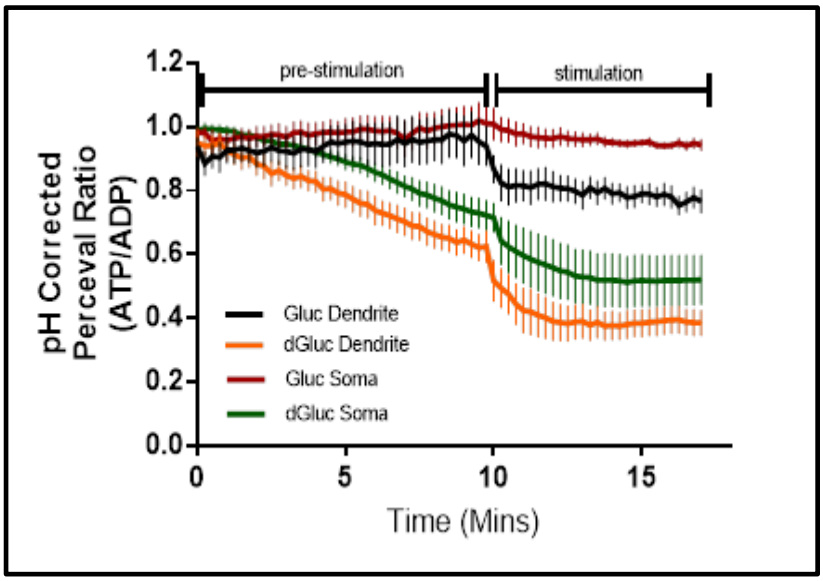


Figure 4: Average time traces for ATP:ADP ratio from neuronal soma and dendrite in presence or absence of deoxy-glucose. N≥5 for all groups. 3 independent experiments.

2. It is noteworthy that most of the mitochondrial genes are nuclear-encoded and many of these mitochondrial proteins are synthesized in the cell body. The relatively smaller neuronal soma and its associated large arbors are both energized by a pool of mitochondria originating only from the soma itself. It is likely, therefore, that stimulation may post a stronger burden to the energy supply within the "energy-hungry" neuronal arbors (24)(26)(27) than within the soma itself.

In our observation, the somatic ATP: ADP ratio showed a dramatic change only in presence of 2-deoxy glucose and the extent of energy consumption was found to be similar to the dendrites (Figure 4). This suggests an inherent difference in energy supply pathways exists between different neuronal compartments. Therefore, we believe, the explanation to this paradoxical observation lies in a distinct energy demand-supply ratio within various neuronal compartments which needs to be explored further empirically. We have discussed these issues in line no 231-233 and 638-642.

Additional concerns byline or fig. number:

Line 106-7: the comparisons in fig 1B are between Glu vs Glu + translation inhibitors. Please compare Glu+translation inhibitors to baseline if you are going to say that the translation inhibitors completely offset the effect of glutamate.

Response: We can provide the details of the comparison but fundamentally we kept only those comparisons that are more relevant to our conclusions. This is to avoid the statistical problem of "**look elsewhere effect**" (28)(29) due to an increase in the non-coverage rate with an increasing number of comparisons for a defined confidence interval. Also, we don't exactly wish to state that protein synthesis inhibitors completely offset energy consumption. We have clarified this in line no. 624-625.

Line 118: The MPEP treatment has not compared to baseline, and the ANOVA info table is not helpful as presented.

Response: In the table of statistical details, we have presented the comparison between basal and glut + MPEP and between glut and glut+MPEP (Kindly refer to the table in figure 1C). We have tested the impact of MPEP treatment separately in supplementary figure 1D. We did not observe any significant alteration in ATP/ATP+ADP tested through one sample t-Test which we have now included in the table of statistical details in the S1D, E, and G sections.

Line 127: NMDA is only 3 data points

Response: Here the experiment was done with 3 batches of independent cultures for all the groups i.e. Basal, DHPG treated and NMDA treated cells and the statistical inference was drawn from the entire dataset.

250-2: Dendritic energy use increases with DHPG treatment, but somatic energy use is not affected. What is the area ratio for the soma vs. dendrites in these neurons, and how does that work with the results from Figure 1 for lysates that include neuronal soma, dendrites, and axons plus of course glia?

Response: Our measurements of the ATP/ADP ratio from the cell lysates in Figure 1 represent an average population behavior of neurons on glutamate stimulation. While quantifying ATP consumption in a compartment-specific manner, we realized that the protein-synthesis dependent ATP usage on DHPG or NMDA stimulation was prominent in the dendritic compartments but not in cell soma. Multiple studies have indicated that in a mature large excitatory cell, dendritic area/volume largely surpasses the somatic area/volume. For example, the dendritic versus somatic morphology parameters can be different by an order of magnitude for pyramidal cells (30)(31). Not just the size, the biosynthetic potential of dendritic arbors largely outgrows that of cell soma (32). Thus, in our understanding the biochemical measurements, which lack the spatial resolution and represent an average behavior of a population, are majorly dominated by dendritic contributions than the somatic. We have, however, ignored contribution from glial cells as the astroglial ATP content is reported to be unaltered on glutamate stimulation (33), which is the major glial cell type we find in our culture.

Another example is the concern I had from the first review of the paper, which is that "protein synthesis [could be] necessary to initiate or

maintain another process that consumes energy, such as transporter activity, vesicle release + recycling, etc." I then suggested the use of a non-canonical amino acid that would allow protein synthesis but prevent the Production of functional proteins. They dismissed my suggestion with three Arguments. The first one was that non-canonical aa's don't affect protein function or location, and this isn't correct because there are NC aa's that inactivate protein functions (see Essays Biochem. 2019 Jul 3; 63(2): 237-266.,Using genetically incorporated unnatural amino acids to control protein functions in mammalian cells). Next, they said the NC aa's would compromise the animals' health, but they are using cell lines and the NC aa's aren't there for days, although since their cells aren't healthy enough to fire spontaneous APs, perhaps the brief incubation with NC aa's would have damaged the cells further. Finally, they said that NC aa's would bias energy utilization to break down the inactive proteins. This makes sense, but it's not clear to me how much protein synthesis was occurring before glu stimulation and if it's enough to cause a significant build-up of proteins before glu stimulation. *Lastly, they said "We will incorporate the possibility raised by the reviewer in the discussion which will no doubt enhance the interpretation of the paper", but they don't provide line numbers and I do not see this point addressed anywhere in the discussion section. I've also looked through the results and can't find it there Either. Because of this, it feels like the authors ignore any alternate. explanation of their work and they resist showing comparisons that don't completely support their hypothesis and this concerns me. *

Response: We apologize for missing to incorporate the discussion regarding the alternate source of energy consumption. This is unintentional. We appreciate the insight put forward by the reviewer and don't intend to respond to her/his queries 'sub-optimally'. We have tried to address the reviewer's concern by part:

"protein synthesis [could be] necessary to initiate or maintain another process that consumes energy, such as transporter activity, vesicle release + recycling, etc."

We really appreciate the call for probing any mechanisms downstream of protein synthesis that may lead to such exhaustive energy consumption. However, we would like to reiterate that we have individually tested vesicle recycling and transporter activity for ionic rebalancing, which did not turn out to have significant contributions. It also leaves us with many other alternate mechanisms that may cause energy drainage. Probing further on these mechanisms will certainly expand our understanding of the energetics of neuronal stimulation, but at the moment this is beyond the scope of the manuscript. Yet we have mentioned these possibilities in line no 620-624 in the discussion section of our manuscript.

"The first one was that non-canonical aa's don't affect protein function or location, and this isn't correct because there are NC aa's that inactivate protein functions"

We thank the reviewer for pointing to this information. We are apologetic for the claim before and have mentioned this information in line no 623-624.

"although since their cells aren't healthy enough to fire spontaneous APs, perhaps the brief incubation with NC aa's would have damaged the cells further."

While there is a possibility that treatment of NC amino acid can damage the health of the cells, through multiple facets of experimentation, we have established that our cells are healthy and fire spontaneous APs as discussed before. However, non-canonical amino acids can have a detrimental effect on neuronal health because of the labile proteins they make (34)(35) and need to be tested for their potential health impacts individually at a comparable time scale of treatment. E.g. AHA incorporation does not lead to significant toxicity in neurons as tested before (36).

"Finally, they said that NC aa's would bias energy utilization to break down the inactive proteins. This makes sense, but it's not clear to me how much protein synthesis was occurring before glu stimulation and if it's enough to cause a significant build-up of proteins before glu stimulation."

As rightly pointed out before and agreed by the reviewer that the burden to clear the non-functional proteins would be too heavy for the cell and can influence its health and firing properties. Since we also don't have a clear mechanistic candidate to inactivate, a large-scale non-functional protein production may affect important protein functions, for example, glycolytic or mitochondrial proteins. We know that there is a significant protein synthesis happening within neurons even at the baseline from our FUNCAT data (Manuscript Figure S3A, compare FUNCAT +/- anisomycin). Thus, the final output of such experiments can, in fact, lead to a more complicated scenario where multiple factors influence energy homeostasis. We think this won't necessarily lead to any conceptual advancement considering the regime of topics we have discussed in the manuscript.

Concerns Related to Reviewer 3:

"I believe the authors scientifically addressed the majority of my raised concerns, as well as those of other authors where I had sufficient expertise to evaluate them, and thus scientifically it is probably now suitable. However, the manuscript still suffers from an excessive amount of typos and grammatical errors. Here are some e.g.'s of typos and writing that could be clarified on the first page:
Line 45: "The brain"
Line 46: "This consumption"
Line 47, 59: Typo: I don't think 'evidences' is used as a plural noun.
Line 50: "Lead" (the relevant noun here is 'Conditions' so the verb is lead, rather than 'leads')
Line 85: "How is activity"
Line 88: "a large amount"
Line 37,55 and throughout: Use of "after" or "upon" rather than "on" here and throughout the manuscript might be a more clear word choice.
Line 94: "after both stimuli"
Line 95: rephrase
Fig 1A: period? Preload?
After Line 95, I've stopped with this kind of editing and focused on scientific review, but the manuscript continues to need deep editing for standard English usage before it will be suitable for publication. However, this is probably more a role for a copy editor than a scientific reviewer. Scientifically I think they addressed my concerns. I would note that in addition to copy editing, it would not hurt to think about the order and framing of the newly added data a bit. For example, the electrophysiological analysis added for one of the reviewers to show the neurons are healthy? For a naive reader not familiar with what the reviewers asked for, it is not really explained why it is there. Perhaps, as it is mostly a control, it might be more of a method section thing anyway.
one last typo - Line 468: "the complex a regulation" - not sure what this is meant to say."

Response: We thank the reviewer for finding the manuscript 'suitable' for publication. We apologize for all the copy editing and labeling related issues. We have now taken the utmost care to correct the typographical and grammatical errors as suggested. We agree with the reviewer regarding the order and framing of the data presentation for which we have made some modification in the overall structure of the manuscript.

We also agree with the reviewer that some additional explanations were required for the new data added later. We have added the necessary details as and when we felt a requirement, for the understanding of the broader spectrum of readers.

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3rd Editorial Decision 6 March 2020

Thank you for the submission of your revised manuscript. We have now received the enclosed comments from referees 1 and 2. As you will see, referee 2 still raises several points that would need to be successfully addressed in order for us to proceed with the handling of your manuscript here.

A few other changes are also required:

Please add up to 5 keywords to your manuscript.

Please use exclusively initials for author names in the author acknowledgement section.

Please send us a conflict of interest statement.

Please correct the reference style, up to 10 authors should be mentioned before "et al" and the journal names need to be italicised.

Fig 4I callout is missing and Fig 6H callout is missing. Please add.
There are callouts to Supp Fig 5, but this figure is not uploaded.
The table callout is missing, please add.

Please add a table of content with page numbers to the Appendix file.

Fig 1I (was G) - the lower 3 bands are overcontrasted.
Fig 2B+C - the scale bars need to be thicker/the numbers need removing.
Fig 3G - the top 3 left bands are overcontrasted.
Fig 4C - the top 2 left bands are overcontrasted. All the right bands are overcontrasted.
Supp S3E (was 2C) is overcontrasted. Please generate better images for these.

Please move the figure legends to the end of the Article file.

The supplementary figures should be either EV figures (we can layout a maximum of 5) or Appendix figures.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript.

I would like to suggest a few changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

While neuronal activity creates a considerable bioenergetic demand of the brain, the exact source of such abundant ATP outlay at the cellular level is still unclear. Using various pharmacological inhibitors, we show that protein synthesis following glutamate stimulation consumes a sizeable proportion of ATP in cultured cortical neurons and outstrips the energetic demand of ionic rebalancing required following depolarization. We observe dynamic alteration of neuronal protein synthesis upon activation of mGluR and NMDAR. The downstream signaling mechanism of both receptors modulates AMP-activated protein kinase (AMPK) function. AMPK alters the phosphorylation of eukaryotic elongation factor 2 (eEF2) to regulate the distinct kinetics of receptor-specific translation. Thus, our study connects individual glutamate receptors to neuronal protein synthesis and metabolic regulation.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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REFEREE REPORTS

Referee #1:

As I stated in my previous review, the authors have satisfactorily addressed my concerns.

Referee #2:

The authors' have demonstrated that they can culture cells that have spontaneous AP activity and AP induced neurotransmitter release. The authors' have also shown that external application of glutamate causes a statistically significant increase in ATP consumption, and that inhibiting protein synthesis by pretreatment with anisomycin significantly reduces the ATP consumption (Fig 2, in the 95041_3_rebuttal document). The authors have addressed my major fundamental concerns about the ability for their cultures to spontaneously fire APs and subsequent synaptic transmission. This was necessary to determine since basal activity levels of the culture will affect energy dynamics in the neurons, and the major point of the manuscript is the finding that the energy consumption that occurs immediately after the application of glutamate is largely due to protein synthesis initiated by glutamate. It would have been best to repeat each condition from the cell culture experiments from figures 1B and 1C in cell cultures that were tested for spontaneous AP activity, but only the experiments from 1B were repeated. In addition, the statistical significance for the ATP consumption under basal vs glutamate treated cells was far lower in the repeated experiments (p=0.005) compared to the original experiments (p,0.0001 ?). However, the finding that there was a statistically significant decrease is sufficient to proceed if the authors will provide this information about the results from high density vs low density cell culture clearly in their results section. Lastly, in the first revision of this paper (~October, 2019), the authors showed electrophysiology data that was not done with cultures at the same cell density. Therefore, the electrophysiology data in figure 1 of revision 1 was not representative data for the cell cultures used in those experiments. It's highly concerning that it was presented as representative electrical activity for those cultures. Therefore, it's very important that the authors' are clear about the differences in activity between high and low density culture. This point is listed below (points 3, 4 and 12) along with some additional final major points that need to be addressed:

Major Points
1. For the data shown in 1B and 1C, it's my understanding that the basal ATP/(ATP+ADP) values were measured in separate plates of cells. This is indicated but not specifically stated in Fig 1A and the description in the methods section. This should be directly stated in the methods section.
2. For the data in figure 1, you are measuring the ATP/(ATP+ADP). The first measurement is ATP alone, which will have some intensity value, let's say 100. On lines 918 to 920 you describe the next measurement as: "This was followed by a step converting ADP to ATP which
was then used to measure the ATP and ADP level together constituting bulk of the
adenine nucleotides." This indicates to me that the second ATP measurement will give at least the same intensity value from the first measurement, plus the new ATP signal from the converted ADP. It's not clear how you get values >1 unless there is a quenching step, or a subtraction step that isn't clearly stated, or some uncorrected photobleaching of the initial signal. Please clarify this.
3. In the rebuttal, you presented data from the high density cultures showing a smaller but statistically significant reduction in the ATP/(ATP+ADP) levels that was, on average, prevented by pretreatment with protein synthesis inhibitor. This was necessary to measure since there were many months, perhaps years, between the original ATP consumption experiments and the electrophysiology experiments. This graph, figure 2 in the rebuttal document, needs to be shown. This can be done as a supplemental figure. ***The following needs to be added to the results section: rebuttal figure 2 must be shown in your manuscript, and it must be stated in the results section that the electrophysiology data you show in figure 1 was done months (or >1 year?) later.
4. In the rebuttal, you made the point that different experiments were done at different cell density levels. Accordingly, the data from the high vs low density cultures needs to be clearly labelled in each figure. It also must be clearly stated in the results section and described in the methods section.
5. Figure 1D: is the voltage clamp trace (lower data trace) a measurement of the ionotropic receptor responses to neurotransmitter release in response to AP activity and transmitter release from other

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neurons, or is the voltage clamp trace really a measurement of bursting APs in the recorded neuron, or both?
6. Figure 1D vs 1F: It looks like the miniature responses are equal to or larger than the responses from the voltage clamped APs (or synaptic responses generated by APs in adjacent neurons)? The current bars are different values, but the length of the current bar in Fig 1D is ~1/3 the length of the bar in Fig. 1F. So if both bars were made to be the same size, the bar in Fig. 1F would be ~33pA, which means the miniature spontaneous activity in this trace looks larger than the AP responses shown in 1D, which is unusual. In addition, it appears that the average mEPSC amplitude found in your experiments is higher than I normally see in other publications. Please comment on this in the results section.
7. Lines 115 to 120, Sup. Fig. 1 A: While DHPG treatment cause a decrease in ATP levels, NMDA also caused a reduction. Although the reduction is not statistically significant, there is still some effect, and given the low n-values (n=3), it's not reasonable to dismiss the effect of NMDA. Please increase the n-value to at least n=5 for baseline, NMDA and DHPG; alternatively, you can change the sentence to directly state that NMDA did reduce the amount of ATP but the value is not significant, and provide the p-value.
8. Lines 138-142, Sup Fig 1 D and E: you show how anisomycin alone, and CHX alone (without glutamate application) affect the ATP/(ATP+ADP) values in basal media. What are the basal levels of ATP/(ATP+ADP) in these experiments. It is very important to also show the basal level of ATP/(ATP+ADP) for these experiments. Please show the basal levels for these experiments.
9. Supplementary Figure Legend 1D, 1E, and 1G: what is an imaginary baseline? Was the basal activity level tested? If not, then the data in Sup Fig 1 D and 1E and 1G are simply qualitative and statistical significance cannot be properly calculated. Please address this.
10. Line 159-161, a concerted increase in mGluR dep protein synthesis was not directly observed at this stage of the paper. The data are consistent with this, but you haven't directly seen protein synthesis being triggered by mGluRs, so you can't say it.
11. Supplementary Fig. 1 L and M do not belong with other data in this figure, and these two panels are not mentioned in the results section of the paper. Please fix this or remove these two panels of this figure.
12. Figure 1 vs Figures 2 to 6: The electrophysiology data showing that spontaneous APs occur and that they can generate synaptic transmission only applies to the data in figure 1 since that figure used a higher density of cells. That the lower density cultures have decreased or absent spontaneous APs, and that these cultures are not active (no spont AP activity and subsequent AP generated neurotransmitter release). This absolutely needs to be directly stated and clearly explained in the results section.
13. Line 226-227 and Figure 2E: anisomycin treatment ATP/ADP. It is possible that the increase in ATP/ADP simply offsets the decrease in ATP/ADP caused by either NMDA or DHPG treatment. In other words, the excess ATP/ADP could be consumed by some process (other than protein synthesis) that is activated by NMDA or DHPG. This possibility needs to be stated in the results section.
14. Fig 3, B,C,D,E, F: The MAP2B fluorescence was used to normalize the FUNCAT signal intensity. How variable was this ratio under resting conditions? In other words, how variable was the labeling intensity for MAP2B and FUNCAT separately, and did the strength of the signals (intensity, high or low) match each other?
15. Figure 3H: In the methods section, please clearly describe your measurements for this graph because similar measurements are shown in several figures in the manuscript.

Minor points:
1. Figure 1 A and 1H, Typo in figure, pre-incubation treatment description: change "preiod" to period.
2. Line 173: the p-value for "****" is not given
3. Line 135-136 vs line 809: what is the concentration of TTX used for your experiments?
4. Supplementary Fig. 1G: not "mPEP"
5. Line 221, figure not figures
6. Line 224, and?
7. Line 328, this is the major validation for looking at eEF2; I personally think it should be emphasized a bit more, but this is completely up to the authors. Absent the red highlighting, it may not be as clear to readers how important this point is.
8. Line 338: fig 3G and 3H
9. Line 339: sup fig 3D is FUNCAT; should say 3E and 3F

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10. Supplementary Figure 3B: define the abbreviations on the x-axis
11. Line 397: should say why "they need" to regulate AMPK activation (ie. to regulate protein synthesis)
12. Line 623, fix sentence wording starting with "lesser"
13. Figure 5 D,E: should include what the dF/F is measuring on y-axis title.
14. Line 1023, missing the rest of the ref. title
15. Line 1104, missing punctuation in title
16. Line 1131: missing senior author's name
17. Line 1132, this ref is for a book series. "Author Manuscript" is not part of the series title, and the article's title is partially missing.

3rd Revision - authors' response 12 March 2020

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Response to Reviewer 2:

We thank the reviewer for the thorough review of our manuscript multiple times and for providing valuable suggestions. We have attached our response to reviewers' concerns as follows:

"The authors' have demonstrated that they can culture cells that have spontaneous AP activity and AP induced neurotransmitter release. The authors' have also shown that the external application of glutamate causes a statistically significant increase in ATP consumption and that inhibiting protein synthesis by pretreatment with anisomycin significantly reduces the ATP consumption (Fig 2, in the 95041_3_rebuttal document). The authors have addressed my major fundamental concerns about the ability of their cultures to spontaneously fire APs and subsequent synaptic transmission. This was necessary to determine since basal activity levels of the culture will affect energy dynamics in the neurons, and the major point of the manuscript is the finding that the energy consumption that occurs immediately after the application of glutamate is largely due to protein synthesis initiated by glutamate. It would have been best to repeat each condition from the cell culture experiments from figures 1B and 1C in cell cultures that were tested for spontaneous AP activity, but only the experiments from 1B were repeated." In addition, the statistical significance for the ATP consumption under basal vs glutamate treated cells was far lower in the repeated experiments ($p=0.005$) compared to the original experiments ($p=0.0001$?) However, the finding that there was a statistically significant decrease is sufficient to proceed if the authors will provide this information about the results from high density vs low-density cell culture clearly in their results section.

Response: We thank the reviewer. We have addressed the remaining concerns in detail in the major points section.

Lastly, in the first revision of this paper (~October 2019), the authors showed electrophysiology data that was not done with cultures at the same cell density. Therefore, the electrophysiology data in figure 1 of revision 1 was not representative data for the cell cultures used in those experiments. It's highly concerning that it was presented as a representative electrical activity for those cultures. Therefore, it's very important that the authors' are clear about the differences in activity between high and low-density cultures. This point is listed below (points 3, 4 and 12) along with some additional final major points that need to be addressed:

Response: We apologize again for this confusion. To clarify, we have used two different cell densities in our cell culture experiments - $2*10^4$ /cm^2 and $4*10^4$ /cm^2 for various observations presented in the manuscript. For the ease of patching from single neurons, initially, we recorded from low-density cultures and provided the representative traces on the first revised version of the manuscript (October 2019). However, as per the suggestion of the reviewer, we recorded again from our cells,

but this time from the high-density culture conditions. Though the electrophysiology parameters changed with the change in the density, our main observation that anisomycin could rescue the effect of glutamate-mediated dip in the ATP levels could still be validated. We have addressed the other concerns in the major points section.

Major Points

1. For the data shown in 1B and 1C, it's my understanding that the basal ATP/(ATP+ADP) values were measured in separate plates of cells. This is indicated but not specifically stated in Fig 1A and the description in the methods section. This should be directly stated in the methods section.

Response: We agree with the reviewer. We have clarified this in methods section line no 732-737.

2. For the data in figure 1, you are measuring the ATP/(ATP+ADP). The first measurement is ATP alone, which will have some intensity value, let's say 100. On lines 918 to 920, you describe the next measurement as: "This was followed by a step converting ADP to ATP which was then used to measure the ATP and ADP level together constituting the bulk of the adenine nucleotides." This indicates to me that the second ATP measurement will give at least the same intensity value from the first measurement, plus the new ATP signal from the converted ADP. It's not clear how you get values >1 unless there is a quenching step, or a subtraction step that isn't clearly stated, or some uncorrected photobleaching of the initial signal. Please clarify this.

Response: The reviewer is correct in stating that "This indicates to me that the second ATP measurement will give at least the same intensity value from the first measurement, plus the new ATP signal from the converted ADP". However, the entire dataset is presented as normalized ATP/(ATP+ADP) ratio values and all the groups are normalized to the average value of the basal group which also allowed us to calculate the variance of the basal group with values obtained from different basal plates. The normalization with average basal value produces data points that are spread around the mean of 1 in case of basal and therefore constitutes normalized values both greater and lesser than 1. However, we have added an explanation about the nature of normalization in the main figure and supplementary figure legends wherever we have used such a method to normalize the dataset. E.g. in figure 1B in the main manuscript we have added an explanatory sentence in the line 1028-1029.

3. In the rebuttal, you presented data from the high-density cultures showing a smaller but statistically significant reduction in the ATP/(ATP+ADP) levels that were, on average, prevented by pretreatment with protein synthesis inhibitor. This was necessary to measure since there were many months, perhaps years, between

the original ATP consumption experiments and the electrophysiology experiments. This graph, figure 2 in the rebuttal document, needs to be shown. This can be done as a supplemental figure. ***The following needs to be added to the results section: rebuttal figure 2 must be shown in your manuscript, and it must be stated in the results section that the electrophysiology data you show in figure 1 was done months (or >1 year?) later.

Response: We have added the data in Figure EV 1M. We have also added the necessary explanations in the result section, in line number 142-148.

4. In the rebuttal, you made the point that different experiments were done at different cell density levels. Accordingly, the data from the high vs low-density cultures need to be clearly labeled in each figure. It also must be clearly stated in the results section and described in the methods section.

Response: We have mentioned the density of neurons for each assay in various sections. E.g. in line number 112 in the results section, in line number 566 in method section and line number 1022-1023 in the figure legends. We have also added this density term in all the other figures and figure legends.

5. Figure 1D: is the voltage clamp trace (lower data-trace) a measurement of the ionotropic receptor responses to neurotransmitter release in response to AP activity and transmitter release from other neurons, or is the voltage clamp trace really a measurement of bursting APs in the recorded neuron, or both?

Response: The voltage clamp trace is a measure of both, the bursting APs characterized by the membrane depolarization and the ionotropic receptor responses to neurotransmitter release shown by the EPSCs.

6. Figure 1D vs 1F: It looks like the miniature responses are equal to or larger than the responses from the voltage-clamped APs (or synaptic responses generated by APs in adjacent neurons)? The current bars are different values, but the length of the current bar in Fig 1D is ~1/3 the length of the bar in Fig. 1F. So if both bars were made to be the same size, the bar in Fig. 1F would be ~33pA, which means the miniature spontaneous activity in this trace looks larger than the AP responses shown in 1D, which is unusual. In addition, it appears that the average mEPSC

amplitude found in your experiments is higher than I normally see in other publications. Please comment on this in the results section.

Response: The reviewer is correct in stating "which means the miniature spontaneous activity in this trace looks larger than the AP responses shown in 1D, which is unusual." There was a labeling mistake on the Y axis scale bar of the main voltage-clamp trace in figure 1D. We have cross-checked and altered to 100pA from 20pA stated earlier. The average mEPSC amplitude in our culture is higher because all the recordings were performed in Mg^{2+} free ACSF. The quantal release of neurotransmitters has been shown to decreases with increasing concentration of Mg^{2+} increases (Dodge and Rahamimoff, 1967) and therefore consistent with recordings done in Mg^{2+} free ACSF (Sibarov and Antanov 2015).

7. Lines 115 to 120, Sup. Fig. 1 A: While DHPG treatment causes a decrease in ATP levels, NMDA also caused a reduction. Although the reduction is not statistically significant, there is still some effect, and given the low n-values (n=3), it's not reasonable to dismiss the effect of NMDA. Please increase the n-value to at least n=5 for baseline, NMDA and DHPG; alternatively, you can change the sentence to directly state that NMDA did reduce the amount of ATP but the value is not significant, and provide the p-value.

Response: We have altered the explanation as per the suggestion in line number 128-130.

8. Lines 138-142, Sup Fig 1 D and E: you show how anisomycin alone, and CHX alone (without glutamate application) affect the ATP/(ATP+ADP) values in basal media. What are the basal levels of ATP/(ATP+ADP) in these experiments? It is very important to also show the basal level of ATP/(ATP+ADP) for these experiments. Please show the basal levels for these experiments.

Response: The basal group has now been added to the graph in Figure EV 1D, E, and G. Analysis has been changed to One-way ANOVA from One-sample t-test. The normalization statement has been added in the figure legend Figure EV 1D, E, and G.

9. Supplementary Figure Legend 1D, 1E, and 1G: what is an imaginary baseline? Was the basal activity level tested? If not, then the data in Sup Fig 1 D and 1E and 1G are simply qualitative and statistical significance cannot be properly calculated. Please address this.

Response: Earlier the data was presented as a fold-change compared to basal. Hence the basal value for each set was already included in the analysis. However, as per the suggestion, we have altered these figures. Kindly refer to major point 9 for details.

10. Line 159-161, a concerted increase in mGluR dep protein synthesis was not directly observed at this stage of the paper. The data are consistent with this, but

you haven't directly seen protein synthesis being triggered by mGluRs, so you can't say it.

Response: We agree with the reviewer. We have modified the sentence in line number: 173-176.

11. Supplementary Fig. 1 L and M do not belong with other data in this figure, and these two panels are not mentioned in the results section of the paper. Please fix this or remove these two panels of this figure.

Response: We have shifted these data to figure EV 2I and J. We have also mentioned the data in the text of figure 2 in line number: 203-212.

12. Figure 1 vs Figures 2 to 6: The electrophysiology data showing that spontaneous APs occur and that they can generate synaptic transmission only applies to the data in figure 1 since that figure used a higher density of cells. That the lower density cultures have decreased or absent spontaneous APs, and that these cultures are not active (no spontaneous AP activity and subsequent AP generated neurotransmitter release). This absolutely needs to be directly stated and clearly explained in the results section.

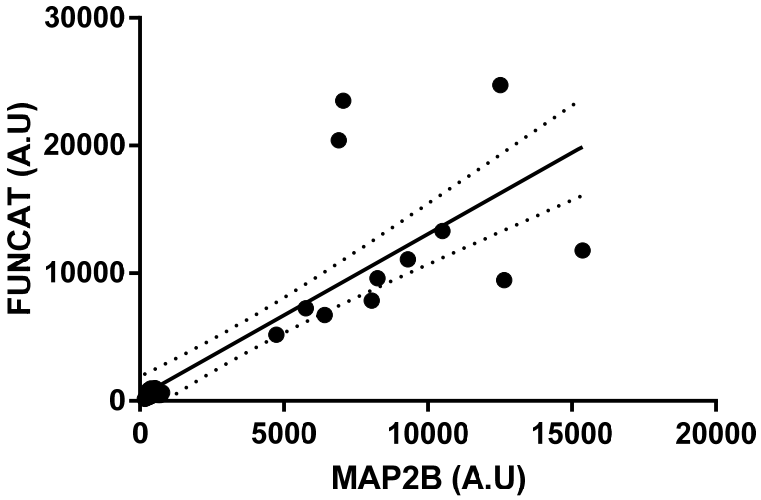
Response: As per suggestions, we have mentioned these observations in the results section of figure 2 in line number: 133 and 186-191. We have also presented the data on the change in spiking frequency with increasing current-steps in low-density neuronal cultures that have been added in Figure EV 1L.

13. Line 226-227 and Figure 2E: anisomycin treatment ATP/ADP. It is possible that the increase in ATP/ADP simply offsets the decrease in ATP/ADP caused by either NMDA or DHPG treatment. In other words, the excess ATP/ADP could be consumed by some process (other than protein synthesis) that is activated by NMDA or DHPG. This possibility needs to be stated in the results section.

Response: This possibility has been added in the results section in line number: 200-203

14. Fig 3, B, C, D, E, F: The MAP2B fluorescence was used to normalize the FUNCAT signal intensity. How variable was this ratio under resting conditions? In other words, how much variable was the labeling intensity for MAP2B and FUNCAT separately and did the strength of the signals (intensity, high or low) match each other?

Response: The FUNCAT/MAP2B ratio has a normalized variance of 0.3046 for the Basal 0 minute group. The MAP2B values are linearly correlated with FUNCAT values in the B0 conditions.



This figure shows the linear correlation between the FUNCAT and MAP2B intensities. As can be seen, most of the data points are within the 95% confidence zone of linearity. This has been tested with Run's Test. The test results as follows:

Best-fit values ± SE	
Slope	1.271 ± 0.1448
Y-intercept	356.7 ± 772.8
X-intercept	-280.5
1/slope	0.7866
95% Confidence Intervals	
Slope	0.9778 to 1.565
Y-intercept	-1211 to 1924
X-intercept	-1794 to 848.3
Goodness of Fit	
R square	0.6818
Sy.x	3854
Is slope significantly non-zero?	
F	77.14
DFn, DFd	1, 36
P value	<0.0001
Deviation from zero?	Significant
Runs test	
Points above line	5
Points below line	33
Number of runs	9
P value (runs test)	0.4555
Deviation from linearity	Not Significant
Equation	Y = 1.271*X + 356.7
Data	
Number of X values	38

Maximum number of Y replicates	1
Total number of values	38
Number of missing values	0

This allows MAP2B intensity to be used for normalization in FUNCAT experiments.

15. Figure 3H: In the methods section, please clearly describe your measurements for this graph because similar measurements are shown in several figures in the manuscript.

Response: This has been added in the method section in line number: 740-744.

Minor points:

1. Figure 1 A and 1H, Typo in the figure, pre-incubation treatment description: change "preiod" to period.
Response: Correction done.

2. Line 173: the p-value for "****" is not given
Response: p-value added in line 1029.

3. Line 135-136 vs line 809: what is the concentration of TTX used for your experiments?
Response: We apologize for the confusion. There was a mistake in the TTX concentration in line 135-136 of the previous version manuscript which has been corrected in line number 151-152 in the current version. The concentration has been clarified in the methods section in line number: 504-505.

4. Supplementary Fig. 1G: not "mPEP"
Response: Correction added.

5. Line 221, figure not figures
Response: Altered to Figure EV.

6. Line 224, and?
Response: Correction added, in line number: 194

7. Line 328, this is the major validation for looking at eEF2; I personally think it should be emphasized a bit more, but this is completely up to the authors. Absent the red highlighting, it may not be as clear to readers how important this point is.
Response: We have added a modest change in explanation as per the suggestion of the reviewer in line number: 273-280.

8. Line 338: fig 3G and 3H
Response: Correction added, in line number: 287.

9. Line 339: sup fig 3D is FUNCAT; should say 3E and 3F
Response: Correction added, in line number: 288.

10. Supplementary Figure 3B: define the abbreviations on the x-axis
Response: Abbreviations added.

11. Line 397: should say why "they need" to regulate AMPK activation (ie. to regulate protein synthesis)
Response: Correction added, in line number: 305-306.

12. Line 623, fix sentence wording starting with "lesser"
Response: Correction added, in line number: 416

13. Figure 5 D, E: should include what the dF/F is measuring on the y-axis title.
Response: Correction added.

14. Line 1023, missing the rest of the ref. title
Response: Correction added, in line number: 825-826

15. Line 1104, missing punctuation in the title.
Response: Correction added, in line number: 920.

16. Line 1131: missing senior author's name
Response: Correction added, in line number: 952-953

17. Line 1132, this ref is for a book series. "Author Manuscript" is not part of the series title, and the article's title is partially missing.
Response: Correction added, in line number: 952

Additional Comments 13 March 2020

Reviewers response to authors' statements and questions:

Authors 1: Earlier the effects of individual protein synthesis inhibitors were presented as
fold changes calculated by making a ratio of (treatment group ATP values)/(basal group ATP values) for each set.

Response 1: I assume you mean that the effects of protein synthesis inhibitors on the response to glutamate were tested, as shown in Fig. 1B? The only place you show the effects of protein synthesis inhibitors alone is in the suppl figure, and it's not shown relative to basal levels for that set of experiments.

Authors 2: The statistical significance was tested considering control value 1 for each case, using
the One-sample t Test, an analysis for testing individual group effects.

Response 2: I assume you mean that the basal condition was assigned a control value of 1. The basal condition should be: lysis -> ATP measurement 1 -> conversion of ADP to ATP -> followed a ATP measurement 2, then making a ratio of ATP measurement 1/(ATP measurement 1 + ATP measurement 2). This is how you've written it.

Authors 3: However, the reviewer has recommended us to do a direct comparison
between the basal and the treatment groups i.e to include the variance of the basal group while comparing.

Response 3: Yes, I have asked you to show the baseline measurement for the experiment when you treated with anisomycin and when you treated with Chx. The protein synthesis inhibited values need to be shown relative to baseline levels. I don't understand what you mean by "include the variance of the basal group while comparing". In the figures, you show something you label as an "imaginary baseline", and I don't know what this means. It's essential to know that aniso or chx are not elevating the ATP levels relative to the real basal levels from the same set or sets of cells.

It is essential to show a direct measurement of the basal level of activity that was present in the cells from these specific experiments. This is not complicated. If for some reason you didn't measure the basal ATP consumption levels for these experiments, then the following experiment needs to be done: Baseline, Aniso alone, Chx alone, glu alone, aniso + glu, chx + glu.

Author 4: This would require us to use the same basal group data twice- 1. in the main manuscript to infer the effects of glutamate stimulation and protein synthesis 2. to asses the impact of individual treatment groups in the supplementary figures. We wanted to clarify if this is fine?

Response 4: It sounds like the basal measurement was not made for the data shown in supl. Fig 1 D (and probably not done in sup fig 1 E and G either). I would need to see all of the raw data, not normalized, to determine the variability in the data. Even then, given the relatively low n-values for

the experiments, the variability would need to be very low, and it doesn't look that way. For example, the range for the basal response in supl fig 1 C is 0.7 to 1.3. There are differences in the amount of variability in the different sets of basal level measurements, so previous baseline data can not be used because none of the other basal data sets are appropriate to use. The basal level needs to be shown: Baseline, Aniso alone, Chx alone.

If you do not have these data, then the following experiment needs to be done:

Baseline, Aniso alone, Chx alone, glu alone, aniso + glu, chx + glu.
This is a straightforward request for information. Your names are on this paper, and this is an essential experiment if you want to clearly show that glutamate application triggers protein synthesis and this consumes more ATP than other neuronal process like those involved in maintaining the salt concentrations, and those involved in neurotransmitter release (although that may be a neuronal compartment issue).

You also need to say how you are normalizing the data for the basal condition, and for the treatments. You state the following in figure 1A: lyse cells, then atp measurement, then convert ADP to ATP, then second ATP measurement (which really measures ADP levels), then normalized to ATP+ADP. In addition, the y-axis of your figures (1B and C plus some supplementary figs) is ATP/(ATP+ADP). This is effectively x/ (x+y) as written. If this is correct, then x/(x+y) should be 1 or <1 if both x and y are positive values. So this must be wrong because you show values >1. I can think of ways that different ratios could be >1, but not the way you've written it. You need to say exactly how you get the ratios and how you normalize the data relative to basal levels. For example, if basal protein synthesis is consuming a measurable level of ATP, then when you inhibit protein synthesis you should get a decrease in the ADP level compared to basal ADP levels. But if you are using the total ATP level in the numerator and denominator, then you are also normalizing each treatment condition relative to its own total ATP level, and you would effectively miss an increase in ATP levels caused by inhibiting protein synthesis (for example).

Authors' Response 17 March 2020

Response to the reviewers' comments:

1. "I assume you mean that the effects of protein synthesis inhibitors on the response to glutamate were tested, as shown in Fig. 1B? The only place you show the effects of protein synthesis inhibitors alone are in the supplementary figure, and it's not shown relative to basal levels for that set of experiments."
   Response: It was tested exactly how the reviewer suggested. The ATP/(ATP+ADP) value of protein synthesis inhibitor-treated plates was divided by the basal plate value of the corresponding experiments to obtain a normalized fraction value. However, adding all the data in the main figure made it extremely cumbersome. We explained this in our first response letter. As per suggestion of reviewer 1 "The author should compare the means of each bar to the hypothetical value 0 (One sample t test or Wilcoxon signed rank test)" we have compared with the value of basal considering it as 1 in each experiment and tested using One-sample t-test. This is a standard method to represent fold change data and do statistical analysis on individual groups in a longitudinal dataset (Laake and Fagerlnd, Research in medical and Biological Sciences; DOI: https://doi.org/10.1016/B978-0-12-799943-2.00011-2, 2015; Liu J., Chai T., et. al., Frontier's in Microbiology, 2016)) and approved by the other reviewers. Also, It is proposed that One-sample t-test is more powerful due to the positive correlation between the basal and treated groups (Tsai and Chen et. al, NAR, 2003). Thus it

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perfectly makes sense to us that the effects of these drugs used in multiple experiments were shown separately.

2. I assume you mean that the basal condition was assigned a control value of 1. The basal condition should be: lysis -> ATP measurement 1 -> conversion of ADP to ATP -> followed a ATP measurement 2, then making a ratio of ATP measurement 1/(ATP measurement 1 + ATP measurement 2). This is how you've written it.
   Response: This is correct. The final data set was normalized to the basal values or the average of the basal group. We have now given a detailed explanation on the normalization procedure within the figure legends section and incorporated all other changes suggested. We have also elaborated on the method of calculating the variance of the basal groups under different experimental conditions in the statistical analysis of the methods section.

3. Yes, I have asked you to show the baseline measurement for the experiment when you treated with anisomycin and when you treated with Chx. The protein synthesis inhibited values need to be shown relative to baseline levels. I don't understand what you mean by "include the variance of the basal group while comparing". In the figures, you show something you label as an "imaginary baseline", and I don't know what this means. It's essential to know that aniso or chx are not elevating the ATP levels relative to the real basal levels from the same set or sets of cells.
   Response: Here is what we did: Let us consider we measured baseline ATP/(ATP+ADP) as 0.65, 0.73, 0.68 and 0.72 in 4 different untreated plates (experiments). In each of these experiments, we have the other three groups i.e. anisomycin alone, glutamate alone and aniso+glut. Values from each of these groups would be divided by the value of the basal for corresponding experiment. In this way, we get normalized values from all the other groups from 4 such experiments mentioned above.
   However, now we have 1 for all basal values for which we have not calculated the variance without which we could not perform ANOVA. So, now we make an average of all the values obtained for the basal, i.e. 0.65, 0.73, 0.68 and 0.72 i.e. 0.695. Now we divided all the values with 0.695 to obtain normalized values of 0.935251799, 1.05035971, 0.978417266, and 1.035971223. From this data set, we calculated the normalized variance or index of dispersion, which was plotted as SEM in figure 1B of the original manuscript. This basically means the variance is normalized to mean or variance over mean. This is a widely used analysis protocol used mostly when different groups have different level of variance that may obscure the major effects of any treatment (Santini E., Klann E et. al, Sci. Sig., 2017)

4. "It sounds like the basal measurement was not made for the data shown in supplementary Fig 1 D (and probably not done in sup fig 1 E and G either). I would need to see all of the raw data, not normalized, to determine the variability in the data. Even then, given the relatively low n-values for the experiments, the variability would need to be very low, and it doesn't look that way. For example, the range for the basal response in supplementary fig 1C is 0.7 to 1.3. There are differences in the amount of variability in the different sets of basal level measurements, so previous baseline data cannot be used because none of the other basal data sets are appropriate to use. The basal level needs to be shown: Baseline, Aniso alone, Chx alone."
   Response: All the basal values were measured and there is actually no imaginary line. The fold response has been presented in comparison to a basal value of 1 where the other values are some fraction of 1 because of normalization. The hypothetical or imaginary 1 value is considered when we for sure know that basal is 1 as the other group is represented as the fold-response of basal. The method of estimating ATP/(ATP+ADP) is a biochemical procedure involving multiple enzymatic reaction. The data from this experiment has large intrinsic variability, which is widely accepted. Therefore, analyzing the raw data is meaningless and is conventionally expressed as normalized levels of fold change (Rhee YH, Chung PS. Et. al., BMC Neuroscience, 2019).

   E.g. this is the dataset of ATP/(ATP+ADP) raw values used for 1B in part.

Basal	Glut	Aniso	Glut+Aniso
0.468631618	0.259624286	0.501741176	0.425517509
0.484796492	0.387308281	0.458329693	0.391918211

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0.39457682	0.250319535	0.414483821	0.353249256
0.457560427	0.280921521	0.338847925	0.403028833

After Normalization the table becomes:

1.038193639	0.554005055	1.070651562	0.907999996
1.074004858	0.798909	0.945406371	0.808418
0.874134671	0.6344	1.050451522	0.895261044
1.013666827	0.61395502	0.74055339	0.880821

However, we separated the effect of anisomycin-treatment alone in an acceptable format (Liu J., Chai T., et. al., Frontier's in Microbiology, 2016) so that the readers could emphasize more on the effect of protein-synthesis inhibitors on glutamate stimulation. The basal variation has been otherwise, added to the data in the recent-most version EMBOR-2019-48037V4 of the manuscript as per the suggestion.

5. "You also need to say how you are normalizing the data for the basal condition, and for the treatments. You state the following in figure 1A: lyse cells, then ATP measurement, then convert ADP to ATP, then second ATP measurement (which really measures ADP levels), then normalized to ATP+ADP. In addition, the y-axis of your figures (1B and C plus some supplementary figs) is ATP/(ATP+ADP). This is effectively x/ (x+y) as written. If this is correct, then x/(x+y) should be 1 or <1 if both x and y are positive values. So this must be wrong because you show values >1. I can think of ways that different ratios could be >1, but not the way you've written it. You need to say exactly how you get the ratios and how you normalize the data relative to basal levels. For example, if basal protein synthesis is consuming a measurable level of ATP, then when you inhibit protein synthesis you should get a decrease in the ADP level compared to basal ADP levels. But if you are using the total ATP level in the numerator and denominator, then you are also normalizing each treatment condition relative to its own total ATP level, and you would effectively miss an increase in ATP levels caused by inhibiting protein synthesis (for example)."
Response: These issues have been addressed already. Kindly refer to response no 3 and 4 for this. We have also elaborated on the response letter for V4.

Additional Comments 27 March 2020

Referee #1

I carefully read over the queries and answers. All concerns seem to be centered around the question of normalization, and I am fully satisfied with the authors' response and way of normalization. In fact, they do more than other authors by taking the extra step of calculating the variance for their basal condition, thereby avoiding the 'error bar-less' control bar one sees in many papers. In my view (and supported by the citations provided in the responses), the authors use a standard way of analyzing data from separate experiments and clearly describe this method in the manuscript.

Accepted 27 March 2020

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ravi Muddashetty
Journal Submitted to: EMBO Report
Manuscript Number: EMBOR-2019-48037V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We chose the sample size based on empirical observation without any prior prediction of effect size as in many cases the effect size is a biological question that we are trying to address. So predicting an effect size with certain level of significance can bring in bias in the dataset.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We have tried keep the sample size btween 4-8 depending on the experiment. We have tried to keep the same number of animals for homogeniety comparison between groups in the same experiment.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We have included animals or cells only when they were healthy. Health was mostly assesed through morphological features. While post-acquisition data analysis, we excluded data points as statistical outlier if the deviation was more than mean +/- 2 S.D. (standard deviations) as they may have created significant bias for the analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	To avoid subjective bias, we tried ensure that experiments are done under identical conditions between groups.
For animal studies, include a statement about randomization even if no randomization was used.	We did not use any randomization method for our animal studies.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For avoiding sampling bias, we tried to increase the sample size to the maximum extent considering the constraints of the experiment. For immunolabeling experiments, we verified the veracity of the observations by doing both double-blining and non-blinding experiments.
4.b. For animal studies, include a statement about blinding even if no blinding was done	We did not use blinding method for our animal studies.
5. For every figure, are statistical tests justified as appropriate?	We believe the statistical tests used in the manuscript figures are appropriate. For comparison of a data set with a predicted value, we used One-sample t test or Wilcoxon-signed rank test. For two sample comparison we used Paired or unpaied T test. For multiple group single variate analysis we used One-way ANOVA followed Bonferroni's multiple comparion tests or Kruskal-Walli's Test followed by Dunn's multiple comparison tests. For multiple group double varible comparisons, we used Two-way ANOVA followed by Bonferrroni's multiple comparison test.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Before using any test, we have always verified the data distribution and checked for normality using Kolmogorov-Smirnov Test and Shapiro-Wilk test. For normal distribution, we used parametric tests for data comparison. For data distribution not normal, we used non-parametric tests.
Is there an estimate of variation within each group of data?	We did estimate the variation of each data set using Browne-Forsythe test and Bartlett's test. We tried to correct for the difference in variance for Unpaired sample t-tests with Welch's correction.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	In multiple group comparisons, the varicances were not-significantly different in most cases. For two sample comparison, we applied Welch's correction when needed.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We have provided appropriate citations and catalogue number for all the antibodies we have used in the manuscript file.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used primary neuronal cultures from Rat cortices.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Rattus norvegicus, Sprague dawly, male animals for synaptoneursome preps on post-natal day 30 (P30). For neuronal cultures, we used female pregnant rats and dissected embryo after at their embryonic day 17-18 (E17-18).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable for our manuscript
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	All the work and protocols are in compliance with the guidelines and approved institutional animal ethics committee.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable for our studies
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable for our studies
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable for our studies
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable for our studies
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable for our studies
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable for our studies
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable for our studies

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Not applicable for our manuscript since we don't have such kind of data for putting in public repository.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We don't have any dataset that is under the scope of being published in these repositories.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable for our manuscript since we don't have such kind of data for putting in public repository.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable for our manuscript since we don't have such kind of data for putting in public repository.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable for our research